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HUMAN HERPESVIRUS-6 IN MULTIPLE SCLEROSIS: ASSAY DEVELOPMENT, IMMUNE RESPONSES AND HOST GENETICS

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ABSTRACT

MS is a chronic inflammatory demyelinating disease of the CNS that implies impaired motor- and cognitive functions. Life expectancy is not severely affected but the disease has substantial negative effects on the quality of life of patients and their relatives. The etiology still remains unknown, but both genetic as well as environmental factors are considered to contribute to disease susceptibility. The importance of environmental factors is supported by findings such as the MS latitude gradient with increasing incidence closer to the poles and from findings of twin studies where the healthy twin in monozygotic twin pairs discordant for disease have a less than 30% risk for developing MS. A viral etiology was first proposed based on findings of local MS outbreaks. However, in recent years the focus has shifted to more common pathogens. HHV-6 is a ubiquitous human herpesvirus that most people have been exposed to. With the cumulative body of evidences for an association to MS, HHV-6 is a strong etiological candidate and the focus of this thesis has therefore been to investigate its role in MS.

The main aim of my PhD thesis was to investigate a mechanism by which HHV-6 might induce breakage of tolerance and subsequent autoimmune attacks against myelin, which is the primary target in MS, by constituting an adjuvant effect. Firstly, we present a new Q-PCR based $TCID_{50}$ read-out method for unequivocal determination of the infectivity of HHV-6 viral stocks. The validation revealed that the new approach is more robust compared to established methods (paper I). Secondly, we show that HHV-6A is not a potent adjuvant as a non-productive infection of HHV-6A in DC reduces IL-8 secretion and reduces the capacity of DC to stimulate allogenic T cell proliferation. However, HHV-6A exposure of DC leads to the up-regulation of HLA-ABC, via autocrine IFN-α signaling, as well as the up-regulation of HLA-DR and CD86, suggesting that DC get partially activated (paper II). To investigate the clinical relevance of HHV-6 in MS, we characterized MS plasma and CSF for viral DNA and MS plasma for antiviral IgG antibodies. We show no significant difference in the frequency of HHV-6 DNA in plasma or CSF (paper III) or in the status or levels of the antiviral IgG response. However, in paper IV we show that several factors previously associated with MS susceptibility are associated with the antiviral IgG response. Carriership of the MS protective allele *HLA-A*02* is associated with lower antibody levels, possibly reflecting efficient cellular antiviral immunity in *HLA-A*02* carriers. The MS risk factor smoking is associated with lower antibody levels, which may reflect a previously shown general decrease in IgG levels in smokers. Women had higher antibody levels, possibly due to a more active general humoral immunity, as previously shown. Finally, in paper V using GWAS SNP genotyping we show that carriership of the allele *HLA-DQA1*05* is associated with higher antibody levels. Furthermore, we provide a list of 31 host genes with suggestive association to anti-HHV-6 IgG antibody status and 29 host genes with suggestive association to antibody levels that contain or lies within 50 kb of the tagging SNP. The most interesting genes are *KSR-2* with suggestive associated to antibody status, and *TRBV5-1*, *CMIP*, *RUNX1* and *MAML3* with suggestive associated to antibody levels. Several of these genes have vital impact on T cell biology and potential importance for steering Th cells into Th1 or Th2 polarization.

To conclude, the results in this thesis provide a robust read-out approach for $TCID_{50}$ assays of HHV-6A. Furthermore, they expand our understanding for interactions between HHV-6 and the cellular and humoral parts of our immune system and reveal novel insights in cellular pathways with potential importance for anti-HHV-6 immunity.

LIST OF PUBLICATIONS

I. **Rasmus Gustafsson**, Elin Engdahl, Anna Fogdell-Hahn. Development and validation of a Q-PCR based TCID50 method for human herpesvirus 6. *Virol J*. 2012 Dec; 9: 311. doi:10.1186/1743-422X-9-311

II. **Rasmus Gustafsson**, Elin Engdahl, Oscar Hammarfjord, Sanjaya B. Adikari, Magda Lourda, Jonas Klingström, Mattias Svensson, Anna Fogdell-Hahn. Human herpesvirus 6A partially suppresses functional properties of DC without viral replication. *PLoS One*. 2013 March;8(3):e58122. doi:10.1371/journal.pone.0058122

III. **Rasmus Gustafsson**, Renate Reitsma, Annelie Strålfors, Andreas Lindholm, Rayomand Press, Anna Fogdell-Hahn. Incidence of human herpesvirus 6 in clinical samples from Swedish patients with demyelinating diseases. *J Microbiol Immunol Infect*. 2013 March; doi.org/10.1016/j.jmii.2013.03.009

IV. Elin Engdahl, **Rasmus Gustafsson**, Ryan Ramanujam, Emilie Sundqvist, Tomas Olsson, Jan Hillert, Lars Alfredsson, Ingrid Kockum, Anna Fogdell-Hahn. HLA-A*02 carriership, gender and tobacco smoking, but not multiple sclerosis, affects the IgG antibody response against human herpesvirus 6. *Accepted with revisions by Human Immunology.*

V. **Rasmus Gustafsson**, Elin E. Engdahl, Emilie Sundqvist, Tomas Olsson, Jan Hillert, Lars Alfredsson, International Multiple Sclerosis Genetic Consortium, Anna Fogdell-Hahn, Ingrid Kockum. Host genetics influence on anti-human herpesvirus 6 IgG status and levels. *Manuscript.*

ADDITIONAL PUBLICATIONS

I. Nicole Marquardt, Martin A. Ivarsson, Kim Blom, Veronica D. Gonzalez, Monika Braun, Karolin Falconer, **Rasmus Gustafsson**, Anna Fogdell-Hahn, Johan K. Sandberg, and Jakob Michaëlsson. The human NK cell response to yellow fever virus vaccination is largely governed by NK cell differentiation independently of NK cell education. *Manuscript*.

II. Hoof, I., C. L. Perez, M. Buggert, **R. K. Gustafsson**, M. Nielsen, O. Lund, and A. C. Karlsson. 2010. Interdisciplinary analysis of HIV-specific CD8+ T cell responses against variant epitopes reveals restricted TCR promiscuity. *J Immunol* 184:5383-5391.

III. Perez, C. L., M. V. Larsen, **R. Gustafsson**, M. M. Norstrom, A. Atlas, D. F. Nixon, M. Nielsen, O. Lund, and A. C. Karlsson. 2008. Broadly immunogenic HLA class I supertype-restricted elite CTL epitopes recognized in a diverse population infected with different HIV-1 subtypes. *J Immunol* 180:5092-5100.

CONTENTS

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
APC	Antigen presenting cell
BBB	Blood-brain barrier
BCR	B cell receptor
CBA	Cytometric bead array
CD	Cluster of differentiation
CIDP	Chronic inflammatory demyelinating polyradiculoneuropathy
CI	Chromosomally integrated
CIS	Clinically isolated syndrome
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTLA	Cytotoxic T lymphocyte antigen
CV	Coefficient of variation
CYP	Cytochrome P450
DAPI	4’,6-diamidino-2-phenylindole
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DPI	Days post infection
EAE	Experimental autoimmune encephalopathy
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
ELISA	Enzyme-linked immunosorbent assay
EBNA	Epstein-Barr virus nuclear antigen
ER	Endoplasmic reticulum
FoxP3	Forkhead box P3
GA	Glatiramer acetate
GBS	Guillain-Barré syndrome
GWAS	Genome wide association studies
HHV-6	Human herpesvirus 6
HHV-7	Human herpesvirus 7
HLA	Human leucocyte antigen
HPI	Hours post infection
IFA	Immunofluorescence assay
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IM	Infectious mononucleosis
IMSGC	International Multiple Sclerosis Genetic Consortium
IN	Intranasal
IV	Intravenous
JCV	John Cunningham virus
JAK	Janus kinase
MAb	Monoclonal antibody

MBP	Myelin basic protein
MLR	Mixed lymphocyte reaction
MMP	Matrix metalloproteinase
MOI	Multiplicity of infection
MS	Multiple sclerosis
MxA	Myxovirus resistance protein A
NAb	Neutralizing antibody
NK	Natural killer
nOD	Normalized optical density
OCB	Oligoclonal band
OR	Odds ratio
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
PML	Progressive multifocal leukoencephalopathy
PMSA	Primary multiple sclerosis affection
PPMS	Primary-progressive multiple sclerosis
PRR	Pattern recognition receptors
Q-PCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RRMS	Relapsing-remitting multiple sclerosis
SNP	Single neucleotide polymorphism
SPMS	Secondary-progressive multiple sclerosis
STAT	Signal transducers and activators of transcription
TCR	T cell receptor
$TCID_{50}$	50% tissue culture infectivity dose
Th	T helper
TNF	Tumor necrosis factor
TLR	Toll like receptors
Treg	T regulatory cell
UV	Ultraviolet
VCA	Viral capsid antigen
VCAM	Vascular cell adhesion molecule
VZV	Varicella-Zoster virus
VGCV	Valganciclovir
VLA	Very late antigen
WTCCC	Welcome Trust Case Control Consortium

1 INTRODUCTION

1.1 MULTIPLE SCLEROSIS

In 1838 Robert Carswell reported observations of lesions in the dissected brain of a deceased individual. Referring to the histopathological picture of the condition with several (or multiple) scars (or sclerosis) of lesions, the disease where named multiple sclerosis (MS). In the central nervous system (CNS) myelin sheaths surround the axons for insulation to enhance the speed of nerve signals. Myelin sheaths consist of densely packed cell membrane extensions of oligodendrocytes. In MS the myelin sheaths and oligodendrocytes are degraded resulting in reduced velocity of nerve signaling between neurons with naked axons. Clinically this can lead to impairment of all functions controlled by the CNS such as motor and cognitive functions, fatigue and bladder control etcetera (reviewed in [1]). MS onset is typically seen between 20 and 40 years of age. Life expectancy is not severely affected [2] but the disease has substantial negative effects on the quality of life of patients and their relatives [3]. The disease also imposes a high economical costs for society due to expensive treatments [4]. Worldwide, at date an estimated 2.5 million individuals are affected, and there is a lifetime risk of one in 400 to get the disease [1]. Furthermore, females have an approximately two times increased risk to develop MS compared to males [5].

1.1.1 Diagnosis and pathophysiology

To set an MS diagnosis the neurologist can use several different techniques apart from clinical examination but in essence CNS lesions and/or positive clinical evaluations must be disseminated in time and space [6, 7]. If only one clinical relapse and/or CNS lesion are observed the diagnosis is clinically isolated syndrome (CIS), but most patients with CIS subsequently convert to MS. After onset, the disease typically follows a relapsing-remitting course (RRMS) with periods of nearly complete recovery. But as the disease progresses the clinical status is aggravated and symptoms are accumulating. Eventually around 65% of patients with RRMS enter a progressive phase, called secondary progressive (SP) MS (figure 1A). In 20% of patients the disease is progressive from onset, called primary progressive (PP) MS. In both SPMS and PPMS progression starts around 40 years of age (reviewed in [8]) suggesting that the pathological events of the progressive phase follow the same course in both phenotypes but symptoms might be subclinical in initial phases of PPMS prior to diagnosis (figure 1B).

Induction of adaptive autoimmune responses such as activation and infiltration of T and B lymphocytes against myelin proteins in the MS brains is believed to be one important mechanism for the demyelination and was described already in 1868 by Jean-Martin Charcot (reviewed by [9]). Even though the importance of an immune component in MS has been questioned [10] there is a cumulative body of evidence that inflammation is central for the disease pathogenesis. This is given by recent advances in MS genetics that link several immune genes to disease incidence [11, 12] and the efficacy of immunomodulatory treatment for the disease. An early developed tool in the diagnosis

of MS is the investigations of oligoclonal IgG bands (OCB). The principal is that paired plasma and cerebrospinal fluid (CSF) samples from the same patient are run on gel electrophoresis. Distinct bands present in CSF but not in plasma are called OCBs, which are seen in a majority of patients, and these band constitute of antibody products from expanded B cell clones [13]. This suggests an accumulation of B cell clones in the CNS that is not seen in the periphery. The involvement of B cells is further supported by findings of formation of ectopic germinal centers in MS brains [14]. Even though B cells seem important the predominant immune cell types in MS lesions are cluster of differentiation (CD) 8+ T cells [15].

Before entering the CNS, immune cells need to cross the blood-brain barrier (BBB) that is formed by tight junctions between the endothelial cells in the capillaries branching into the brain, and thereby separating the CNS from the periphery [16]. However, T cells express the integrin receptor α4β1 (very late antigen (VLA)-4) on their cell surface upon activation via antigen presenting cells (APCs) can then bind vascular cell adhesion molecule 1 (VCAM-1) to facilitate the migration through the BBB. VCAM-1 is expressed on the endothelial cells of the blood vessels upon stimulation by inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and interferon (IFN)-γ [17]. Once inside the CNS the CD4+ T cells need to find their antigen in order to execute their functions. In MS lesions, cytotoxic CD8+ T cells are the predominant immune cell type interacting with cell surface bound human leucocyte antigen (HLA) class I molecules on various glial cell types and neurons, executing cytotoxic actions as seen by granzyme B expression. Demyelination is the hallmark event in MS and therefore oligodendrocytes are thought to be the main target, at least in the early event of disease [15]. However, based on findings from experimental autoimmune encephalopathy (EAE) where adoptive transfer of autoreactive CD4+ T cells alone induced disease, CD4+ T cells are thought to be the driver of disease [18].

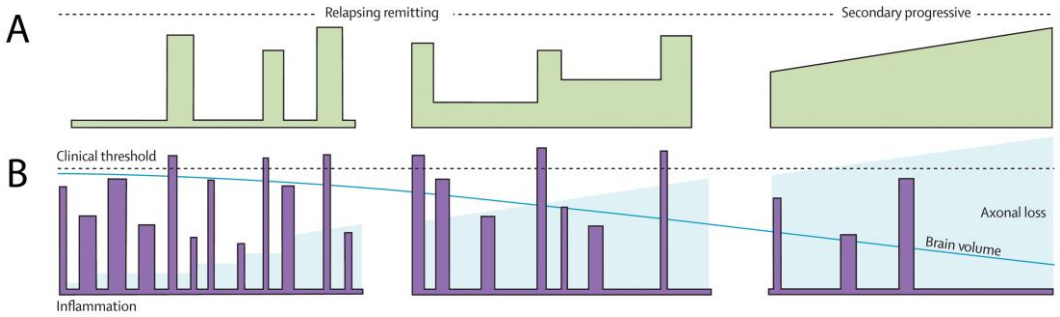

Figure 1. The clinical course of MS. During initial phases of RRMS patients recover to a large extent during the remitting phases but as the disease progresses the clinical status is aggravated and symptoms are accumulating. Eventually around 65% of patients with RRMS enter a progressive phase, SPMS. In 20% of patients the disease is progressive from onset, PPMS (A). The general consensus in the field is that inflammation has the primary role in the initiation of disease followed by axonal loss. The loss of brain tissue such as oligodendrocytes and neurons leads to a reduction of brain volume (B). Reprinted from The Lancet, 372(9648), Compston, A. and A. Coles, *Multiple sclerosis.* p. 1502-17, (2008) with permission from Elsevier.

1.1.2 Risk factors

1.1.2.1 Genetic risk factors

The importance of inflammation in MS was illuminated by genetic findings including the very first identified MS associated gene allele in the 1970s, *HLA-DRB1*15* [19, 20]. It lies within the HLA region that contains genes with crucial roles in the immune system. It would take another thirty years until the next gene allele was identified, *HLA-A*02* [21], also that a gene within the HLA region. Whereas presence of *HLA-DRB1*15* increases the risk of MS around 3 times presence of *HLA-A*02* reduces the risk by half. Interestingly, individuals homozygous for *HLA-DRB1*15* and lacking *HLA-A*02* have a 23 time increased risk for MS [22]. The introduction of genome wide association studies (GWAS), where hundreds of thousands of single nucleotide polymorphisms (SNPs) spanning the entire genome are genotyped, has led to a virtual explosion of newly identified MS associated genes [11, 12]. To date, the list comprises 110 different non-HLA genes associated to MS onset. The various genotypes identified in GWAS studies typically have small effects on susceptibility. In combination with the correction for multiple testing very large materials with tens of thousands of individuals are included to get sufficient power in the statistical analyses.

Hence, genetics seems to play a role. And indeed the unaffected twin in discordant monozygotic twin pairs, whose genetic code is 100% identical, have around 30% risk of developing disease, according to a meta-analysis of populations based surveys [8] (figure 2). A recent and yet unpublished population registry-based Swedish study suggests that this risk is even lower, under 24% (personal communication with Helga Westerlind). The recurrence risk decreases with decreased genetic sharing suggesting a dose effect of genetic sharing that furthermore supports the importance of genetics in MS disease. However, genetics cannot explain the remaining 70-76% of the lifetime risk to get MS. This suggests that environmental factors are of substantial importance, especially as close family members often share environment.

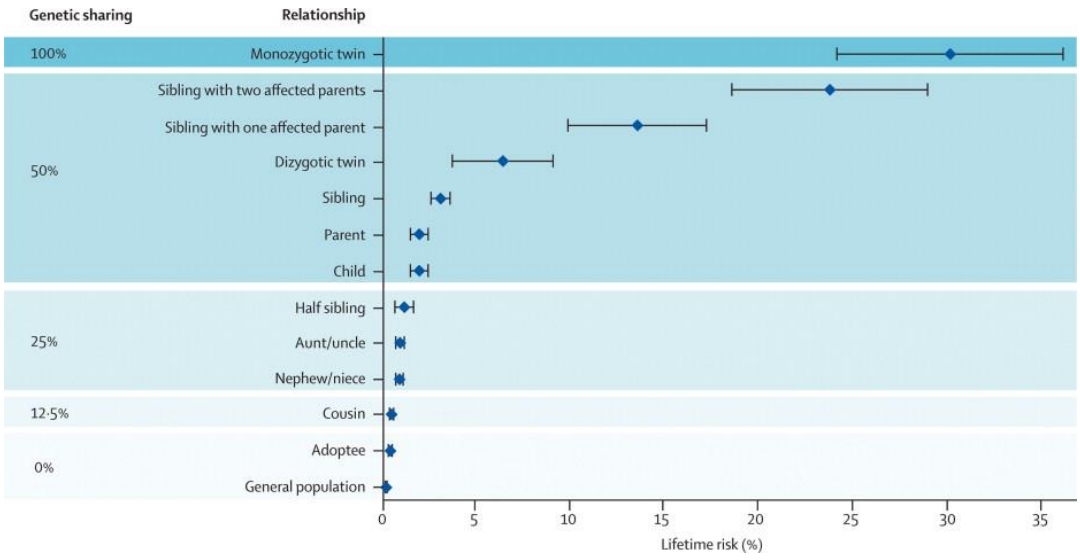

Figure 2. Recurrence risks for relatives to MS patients to get the disease. The recurrence risk increases with increased genetic sharing suggesting that genetics is important. But even with 100% of genetic sharing, as for monozygotic twins, the recurrence risk is only around 30% suggesting that also environmental factors are important. The data is based on population based surveys and error bars represents estimated 95% confidence intervals. Reprinted from The Lancet, 372(9648), Compston, A. and A. Coles, *Multiple sclerosis.* p. 1502-17, (2008) with permission from Elsevier.

1.1.2.2 Environmental risk factors

The role of various environmental risk factors in MS susceptibility is supported by a substantial amount of studies. One interesting phenomenon is that MS susceptibility increases in populations living closer to the poles on both hemispheres [23]. Furthermore, migration from a high-risk area to a low-risk area decreases the incidence of MS, but only if the migration is done before the age of 15 years [24]. The general interpretation of these results is that environmental factors seem to be important in MS, and that the disease is acquired during adolescence.

1.1.2.2.1 Environmental risk factors - non infectious

The most intuitive mechanism underlying the notion of increased MS prevalence closer to the poles is sun exposure and ultraviolet (UV) radiation, which vary with latitude. UV light impact biological processes, it can destroy DNA [25] and cause skin cancer [26]. However UV irradiation also has positive effects such as inactivation of viruses [27] (applied in papers I and II of this thesis) and induction of the conversion of 7-dehydrocholesterol in the skin to pre-vitamin D, which in turn is converted to the vital steroid vitamin D [28]. There is accumulating evidence that vitamin D has a central role in MS. Vitamin D deficiency in blood has been correlated to increase incidence of MS [29]. Furthermore, a vitamin D rich diet has been associated with enhanced recovery from EAE in rat; and a decreased incidence of MS has been seen in people living in coastal areas with a fish based diet compared to people living in the inland of Norway (reviewed in [30]), which has a high MS prevalence (0.16%) [31]. Finally, there is a negative relationship between sun exposure and the risk for first demyelinating events [32].

The cytochrome P450 (CYP) 27B gene has been associated to MS susceptibility [11, 33, 34] and CYP27B is an enzyme that is involved in the transformation of vitamin D to its active form 1,25 dihydroxy vitamin D ($1,25(OH)_2D_3$) [35]. The classical roles of $1,25(OH)_2D_3$ are regulation of calcium and phosphate homeostasis but $1,25(OH)_2D_3$ has been suggested to have additional roles such as effecting differentiation and functions of immune cells [36]. Together the findings of vitamin D deficiency as a risk factor for MS, the MS protective effect of vitamin D rich diets and the genetic association between MS susceptibility and *CYP27B* provide a strong indication that vitamin D is important in MS.

Vitamin D has multiple and complex effects on the immune system but they seem to go in the anti-inflammatory direction (reviewed in [37]). Differentiation of human monocytes to dendritic cells (DC) is inhibited as well as secretion of the pro-

inflammatory cytokines IL-12 and IFN-γ, and in contrast secretion of the anti-inflammatory cytokine IL-10 is enhanced upon stimulation with $1,25(OH)_2D_3$ [38]. The immunosuppressive effect of $1,25(OH)_2D_3$ seems to be even stronger in dermal APCs as the surface expression of co-stimulatory molecules is decreased on dermal DC and as the capacity of these APCs) to stimulate proliferation of allogenic naïve CD4+ T cells is impaired. Furthermore, $1,25(OH)_2D_3$ treated Langerhans cells induce skewing of CD4+ T cells into FoxP3 Treg phenotypes [39]. Vitamin D also seems to have direct effects on human T cells that follow the same path of tolerance induction, as treatment with a vitamin D analogue suppressed proliferation of anti-CD3 stimulated CD4+ and CD8+ T cells, suppressed secretion of the pro-inflammatory cytokines IFN-γ, IL-17 and IL-4, and in contrast enhanced secretion of IL-10 and expression of cytotoxic T lymphocyte antigen (CTLA)-4 [40]. CTLA-4 in turn is a ligand on T cells for the co-stimulatory molecules CD80 and CD86 on APCs that antagonizes the binding of CD28 on T cells, which results in blockage of T cell activation signaling (reviewed in [41]). Given the supports for an anti-inflammatory effect, $1,25(OH)_2D_3$ is on the other hand vital for signaling downstream of the T cell receptor (TCR) in humans [42]. This finding does not necessarily suggest a pro-inflammatory effect of vitamin D and together the literature seems to point in the direction of an anti-inflammatory effect for vitamin D in humans.

As for vitamin D, an immunomodulatory activity is also a possible mechanistic explanation behind the increased MS susceptibility upon active [43] and passive [44] smoking. More specifically an increased activation of T cells by DC has been observed in the lungs of smokers [45]. Another explanation is given by findings of increased expression of matrix metalloproteinases (MMPs) on immune cells and in body fluids of smokers. MMPs are involved in the trafficking of immune cells over the BBB and an increased levels of these enzymes might facilitate the crossing of immune cells, including autoreactive cells [46]. As not all people who smoke develop MS, it is likely that the mechanisms of smoking in combination with other factors are dangerous, from an MS perspective. A link between smoking and infections is provided by a decrease in the general antibody response among smokers [47].

1.1.2.2.2 Environmental risk factors - infectious

The role of a functional immune system is to protect us from infections, and maintain tolerance to self [48]. The hygiene hypothesis assumes that a high exposure of microbes keeps the immune system busy preventing it from attacking body tissues. This is supported by findings including that children growing up on farms were exposed to a wider range of microbes which lowered their risk of developing asthma [49]. The hygiene hypothesis also assumes that infections early in life are handled in a better way when the immune system is more active by providing an efficient immunological memory that optimizes the mechanisms of clearing the pathogen upon exposure later in life. This model has been proposed also for MS. An early Israeli study based on questionnaires suggested that a high hygienic standard at age 10 imposed an increased susceptibility risk for MS later in life [50].

In contrast to the hygiene hypothesis the theory that an infectious agent can trigger MS has been put forward. The immune system seems to be important in MS. But the hypothesis that inflammation is the primary event in MS is based on negative findings. Which is the notion that no causal link has yet been established to any pathogen, as Hafler *et al.* points out in an important review [9]. The potential of a viral cause in MS is suggested by an experimental animal model where infection of susceptible mice strains with Theiler's virus induces immune mediated demyelination [51]. Over the years a long list of different infectious pathogens has been expected to be associated with MS [52]. The idea that MS is infectious came from experiences of several MS epidemics at the Faroe Islands starting in 1943 upon occupation by British troops that were suspected to carry an MS specific pathogen, primary multiple sclerosis affection (PMSA) [53]. However, a potential bias here is that among the British soldiers there might have been clinicians that were the first westerners to observe MS in the Faroe Islands. Hence, the disease might have been present before the islands were occupied, when the natives were not observed by westerners.

Virus infections tend to fluctuate with season and are in general more frequent in populations of the northern hemisphere during the period around October to April [54-56], probably primarily due to our tendency to squeeze together indoor in smaller areas to a larger extent than during summer. Other mechanisms might be the antiviral effect of UV irradiation described above [27] and also that virions maintain their structures better if they are sneezed out in sub-zero Celsius degree environments during winter compared to in warmer environments during summer. Maternal antibodies that are transferred over the placenta during pregnancy [57] gradually degrade and are lost at around six months of age [58]. If MS is caused by a common viral infection early in life, that the vast majority of the population has been exposed to, the handling of the infection is probably the critical event, and not the exposure status. If a vigorous primary infection is a larger risk factor compared to a milder primary infection by the same virus, then one could argue that it would be beneficial to encounter the first winter cascade of viral infections armed with maternal antibodies and spend the subsequent coming summer period to gradually build up an autonomous immune defense. A population based study by Willer *et al.* [59] of MS cases in the northern hemisphere showed that significantly more MS patients were born in May and fewer in November. The data were based on a large study group consisting of over 42 000 MS patients in total to enable detection of the small effects of more patients born in May (9.1%) and fewer in November (8.5%), but still the study contributes an important observation. Given the antiviral effect of UV irradiation, the hypothesis of a causal relationship of a viral infections early in life and development of MS in adulthood, is supported by a study where increased sun exposure during childhood was shown to be associated with decreased MS susceptibility [60].

The relationships between all known human herpesviruses and MS have been investigated. Interestingly, HHV-6 was the only virus found significantly more often in MS patients in a screening of MS patients and healthy controls peripheral blood mononuclear cells (PBMCs) for DNA of seven different human herpesviruses [61].

Even though this thesis is focusing on HHV-6 in MS, Epstein-Barr virus (EBV) should be mentioned. In recent years an accumulating body of evidence for an association between EBV and MS has grown and made it a popular pathogen in MS etiology. The most important findings include increased [62, 63] IgG titers against the Epstein-Barr nuclear antigen (EBNA) 1 in serum from MS patients compared to controls and OCB specificity against EBV [64]. However, the mechanism of EBNA-1 in MS disease has not been elucidated. There seems to be some controversy on whether this increase is seen prior to, or following MS onset. A role for EBV in clinical relapses has been proposed by findings of increased anti-EBV CD8+ T cell reactivity during active phases of MS [65]. The role of EBV in the brain was suggested in an elegant study by Aloisi *et al.* showing infiltration of EBV transformed B cells and formation of ectopic germinal centers in MS brains, and these germinal centers were shown to be the primary site for EBV persistence [14]. Even though this finding has been questioned [66] it remains an important piece of the MS pathophysiological puzzle. Indeed, transformation of B cells by EBV is performed routinely *in vitro* to develop continuous B cell lines. If this occurs in the human body, a transformed B cell clone with B cell receptor (BCR) specificity against myelin proteins would potentially aggravate the pathogenesis. EBV infection often occurs early in life and is then asymptomatic [67]. Primary infection of EBV in adolescents and adults however, can cause infectious mononucleosis (IM) [68, 69] leading to an increased risk for MS in EBV seronegative persons [70], and IM increases the risk for developing MS [71, 72]. Hence, if EBV can cause MS disease, then EBV induced MS onset would occur during adolescence. This is further supported by a study of pediatric MS where a decreased status of IgG antibodies against the EBV proteins EBNA-1 and viral capsid antigen (VCA) was seen in MS cases compared to in healthy pediatric controls [73]. Given these evidences, the possibility that the findings of an EBV association in MS are rather consequences than causes of the disease cannot be ruled out. Therefore, to prove a causal relationship an idea is established within the field that a clinical trial should be conducted where MS patients are treated with anti-EBV drugs [74].

1.1.3 Treatments

Given the inflammatory component in MS the successful treatment used at date is focusing on dampening the activity of the immune system. Interferon (IFN)-β is a first line treatment for relapsing-remitting MS that reduces the relapse frequency by around 30% and the lesion load seen by magnetic resonance imaging (MRI), but does not seem to effect disease progression [75, 76]. Together with IFN-α, IFN-β constitutes the type I IFN family. Short term effects of type I IFN includes the induction of enhancement of antibody production of B cells via CD4+ T cells and the differentiation of CD4+ T cells into IFN-γ secreting Th1 cells, and cross-priming of antigen specific CD8+ T cells (reviewed in [77]). Therefore IFN-β is also an important antiviral cytokine [78]. Indeed, treatment with IFN-β has been shown to reduce the HHV-6A DNA load in serum of MS patients [79]. However, the antiviral effects are not regarded as the primary mode of action for IFN-β treatment. The effects of this cytokine, when administered continually for long periods of time, seem to differ from the acute effects. A subset of the chronic effects of IFN-β treatment, that seems to be responsible for the

dampening of the disease, include suppressive effect on the co-stimulatory molecule CD80 on APC [80] and the potentially reducing capacity of APC to activate T cells, its counteracting effect on IFN-γ signaling [81], its potential to skew the CD4+ T cell repertoire towards a more anti-inflammatory T helper (Th) 2 profile and its inhibitory effect of trans-migration of leucocytes over the BBB [82].

Another first-line treatment against MS is glatiramer acetate (GA). It constitutes a random polymer of four amino acids found in myelin basic protein (MBP) that was initially intended to be used for induction of experimental autoimmune encephalomyelitis (EAE), but instead was fond to reduce EAE in rhesus monkeys after initiation and onset of symptoms of EAE [83]. A tolerance inducing effect of GA on DC has been suggested by findings of decreased TNF and IL-12 production, and increased IL-10 production by DC from GA treated MS patients, compared to untreated MS patients [84]. Treatment with GA is similarly effective as with IFN-β [85].

Natalizumab is a second line treatment in MS. It is a monoclonal antibody (MAb) specific to VLA-4 [86]. It antagonizes VLA-4 and thereby prevents the infiltration of lymphocytes into the brain, and also other tissues. Natalizumab is more efficient than IFN-β in reducing white matter lesions and relapse risk but longitudinal monitoring of treated patients has revealed a horrifying side effect. VLA-4 is expressed on all activated T cells, independently of its TCR specificity. Hence, not only myelin specific but also T cells specific to different viruses are prevented to enter the brain. John Cunningham virus (JCV) is a common virus with a seroprevalence of 44-92% in the normal population depending on geographical region [87], and MS patients seems to be exposed to a similar extent [88]. Reactivation is normally kept in check by immune surveillance of leucocytes roaming the tissues. In a small number of natalizumab treated patients (2.1 cases per 1000 treated patients in February 2012 [89]) led to reactivation of JCV and development of progressive multifocal leukoencephalopathy (PML). The prevention of immune surveillance is suggested as a causative mechanism due to a decrease in general serum IgG load, in addition to the the block of T cells, and increased reactivation of other common latent viruses such as HHV-6 [90].

Parental administration of therapeutic IFN-β and natalizumab can induce the development of neutralizing anti-drug antibodies (NAbs). It is an obstacle that has been shown to block the efficacy of both IFN-β [91] and natalizumab [92] but does not seem to be a major problem for GA. Given the differences between these three established MS treatments, a common theme is their immunomodulatory capacities. Whereas natalizumab, and possibly also IFN-β, prevents influx of immune cells in the CNS, the mode of action for GA seems to be by inducing anergy.

1.2 IMMUNITY AND AUTOIMMUNITY

1.2.1 T and B cells

Anergy and tolerance is the counterparts of immune activation and autoimmunity respectively. An efficient immune system that can protect us from pathogens need the capacity to elicit strong responses to kill the invading pathogen before it replicate and

multiply beyond control. The actions of our immune system are powerful so we need the immune cells to be specific to target only foreign proteins to avoid reactivity against our own body cells and tissues.

The processes of negative and positive selection during T cell development in the thymus prevent autoreactive T cells to enter the peripheral blood [93]. In the classical model the strength of the interaction between major histocompatibility complex (MHC) molecules with bound self-peptide, and the TCR determines the faith of the T cell. If the interaction is too weak the T cell dies by neglect, and if the interaction is too strong the T cell dies via induction of activation [94]. Intermediate interaction, on the other hand, saves the T cell from apoptosis. A classification of two different T cell lineages is defined by the constitution of their TCR. One lineage is called αβ T cells, simply as their TCR are constituted by the alpha (α) chain together with the beta (β) chain, and αβ T cells constitute the vast majority of T cells in the circulation. Another lineage is called γδ T cells as the gamma (γ) and delta (δ) chains constitute their TCR. They are less common in the circulation and are mainly located at sites in the body that are exposed to the exterior, such as in the epithelia of the gut and the skin (reviewed in [95]). The αβTCR domain on αβ T cells can bind MHC molecules on different cell types during different stages of T cell development and immune responses. During development the αβTCR complex binds to MHC molecules on thymic epithelial and thymic APCs, and during activation to MHC molecules on peripheral APCs. During execution of effector functions, the αβTCR variable chain complex on CD4+ T helper cells bind to MHC class II molecules on B cells, and on cytotoxic CD8+ T cells to MHC class I molecules on target cells such as virus infected cells (reviewed in [96]). B cells develop in the bone marrow but before they enter the circulation they also undergo selection processes. B cells that bind to self-antigens with their BCR are either cleared via apoptosis or subjected to rearrangement of the variable regions of their BCR (reviewed in [97]).

Despite the sophisticated control mechanism in T and B cell development, autoreactive T and B cells can be found in the circulation of healthy individuals [98-100]. But why does the immune system of some people fail to keep these autoreactive cells in check potentially inducing the development of autoimmune disease? For MS this is yet unknown. A popular mechanism for immune control is found in the Tregs. They are defined by their expression of the protein forkhead box P3 (FoxP3) and can be further subcategorized into CD4+, CD8+ and natural killer (NK) cells. Their function to actively suppress other lymphocytes is suggested by findings of autoimmune disease development in animals with *FoxP3* gene deletion or FoxP3 cell depletion (reviewed in [101]).

1.2.2 Dendritic cells

T and B cells might mediate immune reactions but they are under control of DC. DC are a heterogeneous family of cells with specialized antigen presenting capacities (reviewed in [102]). They can engulf extracellular compounds such as virions which are subsequently degraded in lysosomes. Proteins of the virus ‘lysate’ are then cut and

degraded into peptides by the proteasome and transported to HLA molecules in the endoplasmic reticulum (ER). For HLA class I restricted peptides this transport typically occurs via the transporter associated with antigen processing (TAP) complex. The peptide containing HLA molecule can interact with the TCR on the T cell surface. In general, HLA class I molecules present short peptides [~9 aminoacids (a.a.)] from endogenously produced proteins and HLA class II molecule present long peptides (10-30 a.a.) from external proteins. DC respond to microbial stimuli, such as pathogen-associated molecular patterns (PAMPs) that are bound by pattern recognition receptors (PRRs) such as toll like receptors (TLR) (reviewed in [103]). In addition, DC respond by inflammatory stimuli, such as inflammatory cytokines. Microbial and inflammatory stimuli can initiate a process of cellular activation in DC termed maturation. The process of maturation is associated with increased surface levels of HLA and co-stimulatory molecules, as well as enhanced production of soluble inflammatory mediators such as type I IFN, interleukin (IL)-8, IL-6, tumor necrosis factor (TNF) and IL-12 (reviewed in [104]).

In the orchestration of T cells, DC interact with the TCR on the T cell using its HLA molecules, typically with a bound non-self peptide. The CD4 or CD8 molecules stabilize the interaction to HLA class I and II molecules respectively. Co-stimulatory molecules CD80, CD83, CD86 and CD40 on the DC bind their ligands CD28 and CD40L on the T cell to further strengthen the interaction (figure 3). Now the DC and T cell have formed a so called 'immunological synapse' and signals will be transmitted to the T cell [105]. Together with B cells and macrophages, DC constitute the group of APCs. They have a special ability to activate CD4+ T cells, via HLA class II molecules, and also CD8+ T cells, via HLA class I molecules, in a process called cross-presentation. Viruses often hijack the replication machinery of the host cell. Hence, the virus infected cell display viral peptides on its HLA class I molecules to allow interaction with CD8+ T cells that induce apoptosis of the infected cells and thereby preventing the virus, such as HHV-6 to spread to neighboring cells.

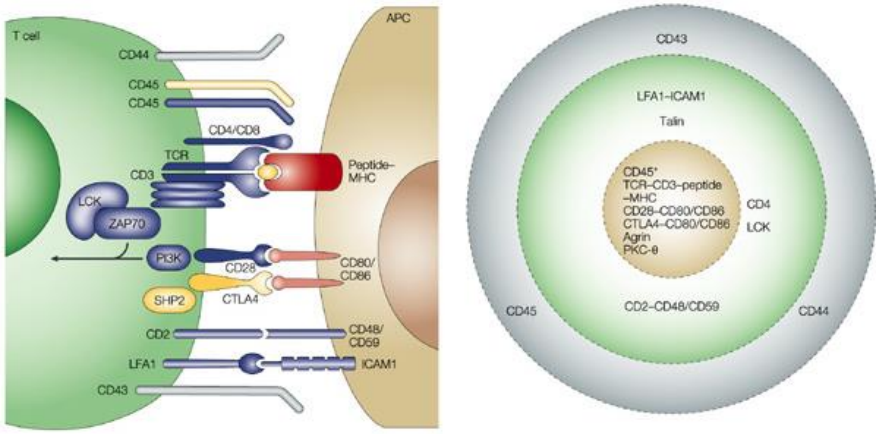

Figure 3. An 'immunological synapse' between a DC and a T cell. Reprinted from Huppa, J.B. and M.M. Davis, *T-cell-antigen recognition and the immunological synapse.* Nat Rev Immunol, 2003. **3**(12): p. 973-83 with permission from the Nature Publishing Group.

1.3 HUMAN HERPESVIRUS 6

HHV-6 was first isolated in the mid 80's from patients with lymphoproliferative disorders and acquired immunodeficiency syndrome (AIDS) [106-108] and belongs to the β-herpesvirus subfamily of *Herpesviridae*, as defined by the Baltimore classification system of viruses. As the other human β-herpesviruses, cytomegalovirus (CMV) and human herpesvirus 7 (HHV-7), HHV-6 can also establish lifelong latent infection in the host. HHV-6 isolates are classified into two distinct virus species, HHV-6A and 6B [109]. The two viruses share 90% of their nucleotide sequence [110]. In this thesis, HHV-6 is used when no such discrimination has been made. Its genome consists of an approximately 160 kilo base pairs (kbp) (159 321 bp for HHV-6A [111] and 162 114 bp for HHV-6B [110]) double stranded DNA containing 97 genes. The viral DNA is surrounded by a core, which in turn is enclosed by an icosahedral capsid. Finally, a lipid bilayer constitutes the outermost barrier to the exterior. This bilayer is from a host cell compartment and is acquired during viral replication and budding from the infected cell.

1.3.1 Epidemiology

At the age of two to three years, most individuals have been exposed to either HHV-6A or HHV-6B. This is supported by an observation of HHV-6 DNA presence in 77 cumulative percent in saliva from children sampled weekly during the first 24 months of life [112] and by the detection of anti-HHV-6 IgG antibodies in 100% of children tested at the age of 16-21 months [113]. As expected, the antibody prevalence decreased during the first five months of age but gradually increased and at 16 months of age 70-100% were positive [114]. The decline in anti-HHV-6 IgG antibody titers was reversibly followed by an increasing frequency of children with positive polymerase chain reactions (PCR) for HHV-6 DNA reaching a 70% plateau at 10-12 months of age (figure 4) [115]. Others have found this plateau at three years of age with a seroprevalence of over 90% and a gradual decline after 40 years of age [116], possibly reflecting a decreased activity of the aging immune system [117].

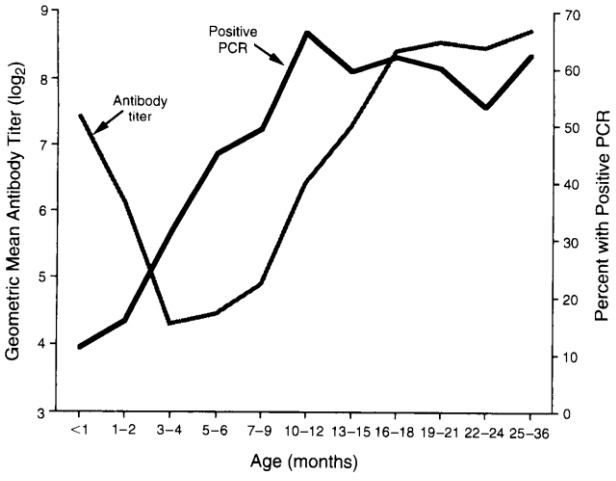

Figure 4. Anti-HHV-6 IgG antibody titers decline during the first four months of age and is reversibly followed by an increased frequency of children with HHV-6 DNA positive PCRs in PBMC. 2427 children were tested without acute illness. Reprinted with permission from Hall, C.B., et al. N Engl J Med, 1994. **331**(7): p. 432-8. Copyright Massachusetts Medical Society.

Whereas HHV-6B seems to be the predominant variant in Europe, Japan and USA [118-120], HHV-6A infection account for the vast majority of active HHV-6 infections

in sub-Saharan Africa [121]. Epidemiological reports on HHV-6 from the African continent are limited, but a study from West Africa report a similarly high seroprevalence as in other populations studied [122]. If the results from West Africa can be extrapolated to sub-Saharan Africa HHV-6A infection in sub-Saharan Africa may be as common as HHV-6B infection in the western world. To wildly speculate, one mechanism behind this misallocated distribution might lie in different capabilities of the viruses to escape immune responses. Whereas HHV-6B is able to evade type I IFN mediated immune defenses by shutting down the production of both IFN-α and -β HHV-6A does not have that same capacity [123]. Upon hepatitis C infection African Americans, with a more recent African heritage than Caucasians, are more resistant to IFN-α therapy than are Caucasians [124]. This has been suggested to be linked to a SNP in the *IL-28B* (IFN-λ3) gene [125]. Interferon-α and -λ3 both signal via the Janus kinase (JAK) – signal transducers and activators of transcription (STAT) pathway but they utilizes different cell surface receptors [126, 127] and data from a recent study suggest that IFN-λ signaling can compensate for a reduced responsiveness to IFN-α [128]. Therefore, HHV-6A might have a better chance to establish infection in populations of sub-Saharan Africa than in the other populations mentioned above. The predominance of HHV-6B in western countries and Japan is harder to interpret. One possibility is that replication by HHV-6A is more efficient than that of HHV-6B, and when type I IFN is not present HHV-6A takes over the scene. Another possibility is the analogy to the type I IFN story; that HHV-6A has an immune evasion strategy, such as suppressing secretion of a certain cytokine, that HHV-6B lacks and that this specific compartment of the immune response is more important for African populations than for Caucasians.

1.3.2 Basic biology

1.3.2.1 Transmission

A major route of transmission for HHV-6 is via the saliva where it is shed [116] but congenital transmission is also important and occurs in 1% of births [129]. This can be acquired by transmission of chromosomally integrated (ci) HHV-6 in the germline [130] and also by reactivation from ciHHV-6 positive mothers to their ciHHV-6 negative children via the placenta [131]. A route of transmission to the CNS has been identified in the olfactory tract [132].

1.3.2.2 Tropism

A cellular receptor for both HHV-6A and HHV-6B is CD46 [133], a complement inhibitory molecule that is expressed on all nucleated cells [134]. Therefore, the viruses may have tropism for many different cell types, including immune cells. A predominant tropism is seen for CD4+ T cells [135, 136], but also CD8+ T cells [137, 138], NK cells [139], monocytes [140, 141], macrophages [140], and DC [142-146]. Moreover, HHV-6 also has neurotropism and can infect neurons [147] and glial cells [107], including astrocytes [148], oligodendrocytes [149, 150], and microglia [150]. The tropism differs slightly between HHV-6A and 6B. CD134 was recently identified as a cellular receptor exclusive for HHV-6B [151]. A specific characteristic of the

dissemination of HHV-6A but not of HHV-6B includes its ability to induce cell-to-cell fusion via CD46 independent of virus replication. This was observed in Chinese hamster ovary (CHO) when they expressed human CD46, and CHO cells are normally highly resistant to HHV-6 infection, [152]. HHV-6A seems to be more neurotropic than HHV-6B. *In vivo* this is seen by a higher frequency of HHV-6A DNA in CSF than in PBMC in children, HHV-6B display the opposite pattern [153]. *In vitro* this is supported by the capacity of HHV-6A but not 6B to induce apoptosis in neurons, astrocytes and oligodendrocytes [154]. An increased tropism for human oligodendrocyte (M03.13), human astrocytoma (U373 MG), and human neuroblastoma (SK-N-SH) cell lines [155], and also progenitor derived astrocytes [156, 157], has also been observed for HHV-6A.

1.3.2.3 Latency

Given the ubiquitous nature of HHV-6 [112] and the frequent findings of reactivation in immunosuppressed individuals such as bone marrow transplant recipient patients [158, 159] most people that have ever been exposed to the virus are likely to be latently infected. Current data suggests that sites of latency from which HHV-6B can be reactivated are hematopoietic stem cells (reviewed in [160]). For HHV-6A the site of latency from which it can reactivate has not yet been determined but is has been found to persist in the CSF of children after the decline of a primary infection [161] suggesting the CNS as a possible site. Human herpesviruses normally achieve latency by covalently closed circular episomes [162-164]. It is possible that this also occurs during HHV-6 latency but it remains to be further investigated (reviewed in [165]). However, it is known that the HHV-6 genome is able to integrate its genome in the telomeric regions of host cell chromosomes [166], termed ciHHV-6. It occurs in 0.2-2% of the population in UK, USA and Japan (reviewed in [167]). HHV-6A has been shown to be able to reactivate *in vitro* [168] and both HHV-6A and HHV-6B *in vivo* [131] from this chromosomally integrated state. This capacity is unique among human herpesviruses.

1.3.2.4 Interactions with the immune system

Another outstanding capacity of HHV-6 is its ability to spread in nearly the entire human population. This is probably, at least to some extent, achieved by the mild clinical phenotype of HHV-6 infection. A virus that immediately kills its host will have difficulties to spread, but a less aggressive virus inducing mild or even subclinical symptoms probably spread more efficiently from one, relatively healthy and mobile individual to another. Other beneficial characteristics of HHV-6 include its capacity to modulate the immune response. T cell proliferation is inhibited by HHV-6 infection by mechanism such as cell cycle arrest in the G2/M phase and induction of the anti-inflammatory cytokine IL-10 [169, 170]. Suppression of IL-2 transcription and translation are other mechanisms that might explain the anti-proliferative effect of HHV-6A [171], as well as the suggested suppressive effect of both HHV-6A and HHV-6B on LPS and IFN-γ mediated IL-12 secretion by macrophages and DC [172, 173]. However, the findings on IL-12 secretion are controversial as we and others have not

seen this effect in DC [174, 175]. This is discussed in the results and discussion section of paper II. Ligation of certain domains of CD46 has been shown to suppress T cell proliferation [176]. CD46 is a cellular receptor for HHV-6 and therefore it is possible that ligation of HHV-6 to CD46 has a role in the suppression of T cell proliferation upon HHV-6 infection. However, it remains to be investigated if HHV-6 utilizes the domains associated with suppressed T cell proliferation or other domains that is not associated with this effect.

1.3.3 HHV-6 diagnostics

When attempting to determine the prevalence and/or titer of a certain virus infection in a population or *in vitro* experimental models it is crucial to carefully choose the methods for sampling and laboratory analyses. This will be discussed in this section.

1.3.3.1 Active infection

The ultimate proof of an active infection with a certain virus is to isolate the virus. This is done by inoculating uninfected and susceptible cells (if available for the specific virus) *in vitro* by a biological sample, such as plasma, from the individual to be tested. A positive result is if the uninfected cells shows signs of infection such as syncytia formation or lysis, or signs of supported viral replication such as translation of viral proteins or transcription of viral messenger ribonucleic acid (mRNA). However, HHV-6 virus isolation is a very challenging and time-demanding process. Alternative approaches include screening of plasma samples for cell free HHV-6 DNA by nested PCR. Plasma rather than PBMCs should be used [177] as HHV-6 can go into latency, possibly in blood cells. As most people are likely to be latently infected a positive PCR signal from PBMCs might be a reflection of a latent rather than an active infection. One problem with screening of plasma for viral DNA with PCR is that the results are only qualitative. A positive signal might be picked up from lysed PBMC leaking viral DNA [178], and not necessarily from free virions. This is further supported by a comparison where virus culture could be established in only 84% of samples containing HHV-6 DNA [179].

Reverse transcription (RT) PCR of HHV-6 mRNAs from PBMCs transcribed at late stages of viral replication and coding for structural proteins crucial for the formation of virions might be a more specific and strict approach, as transcripts of the late genes U31 and U39 were found in 91-96% of PBMC samples from children in acute stages of exanthem subitum where the virus had been isolated, and in no sample from children in the convalescent phase [180]. A third possibility when targeting viral DNA is real time quantitative PCR (Q-PCR) on plasma samples. This method gives a quantitative measure on the amount of HHV-6 DNA present and Q-PCR has been suggested to enable discrimination between ciHHV-6 and active infection. In individuals with ciHHV-6 every cell in the body contains viral DNA [165] and hence the viral DNA loads are typically high, between 3.5 and 5 $\log_{10}$ copies per milliliter (copies/ml) plasma. However, these levels overlap completely with the levels in children with acute infection where the DNA loads range between 3 and 6 $\log_{10}$

copies/ml plasma or serum [177]. Therefore, Q-PCR should be performed on PBMC samples as well. Whereas people with ciHHV-6 would have around 1 viral genome copy per leukocyte, people without ciHHV-6 experiencing a primary infection or a reactivation would have substantially lower viral DNA loads in their PBMC. Since the viral genome is incorporated in every cell in the body, there is a 50% chance that ciHHV-6 is inherited from parent to offspring (reviewed in [167]). So, how do you assess primary infection in individuals with ciHHV-6? At date the method of choice seems to be sequencing of HHV-6 PCR products of variable regions in the viral genome such as glycoprotein B [181] from samples of plasma and of purified PBMC from the very same individual. If the sequences are identical there's probably leakage of ciHHV-6 but if they are not identical a primary infection might be at play. This was performed by Gravel et al. [131] when showing that transplacentally acquired HHV-6 from mothers with ciHHV-6 to their ciHHV-6 negative children can originate from the transmission of reactivated ciHHV-6.

In adults active HHV-6 infection is highly transient and cell free viral DNA is rarely detectable [182]. Possibly because HHV-6 spread largely via cell-to-cell contact [146, 183], instead of releasing large amounts of virions which is the case for many other viruses. Therefore, other strategies can be used where immunological responses to the virus, which are more long lasting, are measured instead. Secretion of antiviral immunoglobulin (Ig) M antibodies by B cells is an early event in an antiviral immune response. In primary infection and reactivation of HHV-6 IgM antibodies were detected five to seven days after onset of exanthema subitum and lasted for up to two months [184]. Compared to HHV-6 DNAemia, which seems to last for only a couple of days, this is a large time window. Detection of IgM by immunofluorescence assay (IFA) or enzyme-linked immunosorbent assay (ELISA) may consequently be regarded as indications of recent primary infection, or more rarely reactivation as IgG antibodies with higher affinity are normally present in these cases. The biological role of IgM antibodies includes the ability to bind foreign antigens or pathogens that the body has never been exposed to [185]. Later in the humoral immune response a shift in the antibody repertoire occurs [186] with affinity maturation via somatic hypermutation of the variable chain coding DNA and isotype switching in germinal centers, resulting in secretion of high affinity IgG, IgA and IgE antibodies. One problem with measuring IgM antibodies is that IgM production requires a relatively strong inflammatory response and is not always detectable upon HHV-6 reactivation [187]. In summary, in assessment of active HHV-6 infection various PCR techniques can be utilized for detection of viral DNA or mRNAs, or serological techniques measuring IgM antibodies.

1.3.3.2 History of infection

Given the various difficulties to detect active infection with HHV-6 described above, alternative strategies might be applied such as investigating the infection history. Virus specific IgG antibodies play important roles in antiviral immunity. Their binding to virions results in neutralization where the antibodies constitute a steric hindrance preventing the attachment of virions to permissive host cells. Another result of the

binding is that the virions are opsonized to enhance that they are engulfed by phagocytosing immune cells such as macrophages.

Children seroconvert upon primary HHV-6 exposure [115] and as the vast majority of the population over the age of two years has been exposed to the virus discrete data on seroprevalence is not very informative. Instead, assessing the titers of antiviral antibodies might give a hint on how strong a primary infection has been; or how strongly and/or frequently the virus has reactivated. This is done for infection with Varicella-Zoster virus (VZV) where the antiviral IgG titers increase during the convalescent phase [188]. Another example is EBV where symptomatic reactivation is associated with higher titers of anti-VCA IgG [189]. In vaccinology repeated inoculations of the vaccine are performed to boost the immune response by increased plasma cell counts and antibody titers. An analogy to this concept is found in the MS field where NAbs against therapeutic IFN-β develop in a subset of MS patients, and these NAbs have been shown to persist in RRMS patients after discontinued treatment, especially if the titers are high [190]. This suggests that repeated exposure to the antigen (virus, vaccine or protein) may increase the IgG antibody titers. Therefore, the antiviral IgG titer might reflect the number and/or magnitude of reactivations of the virus in a certain individual, and serve as an indication of HHV-6 infection history. An important aspect though is that serum anti-HHV-6 IgG titers should be considered as an indication of reactivations or primary infections in the past, and not as an adequate marker for active infection. Indeed, several studies have failed to correlate IgG prevalence and titers with active infection as measured with cell-free HHV-6 DNA [191, 192] or virus isolation [118, 193].

1.3.3.3 Titration methods

If the presence and titers of antiviral IgG antibodies is a sign of history of infection and the detection of virus DNA is a direct measure of active infection and one step closer the actual virus neither of them answers the question on the amount of infectious virions in a blood sample or in an virus infected cell culture. The classical 50% tissue culture infectivity dose ($TCID_{50}$) developed in 1938 by Reed and Muench [194] still holds. This method is applied for many different viruses. For the read-out of HHV-6 infection there are different approaches available. The established read-out methods includes ocular inspection for cytopathic effect (CPE), i.e. enlargement of the infected cells [135], and IFA where the inoculated cells are stained with an antibody against a viral protein and inspected in a fluorescence microscope [195]. IFA is also used for calculation of the infectious units, i.e. the fraction of infected cells [196]. The CPE approach is problematic as some HHV-6 susceptible cells can enlarge even when not infected.

1.3.4 HHV-6 in multiple sclerosis

Whereas HHV-6B is the causative agent of exanthema subitum in young children [197], no disease has been clearly linked to HHV-6A. However, associations between HHV-6, and HHV-6A infection more specifically, and MS have been suggested by

numerous reports. A selection of these include findings of increased prevalence of HHV-6 DNA [198] and protein expression [147, 199] in MS plaques compared to in normal appearing white matter. A role for HHV-6 in the brain is further supported by increased frequencies and titers of anti-HHV-6 IgG [200, 201] and IgM [202] antibodies in CSF of MS patients compared to controls; and OCB specificity against HHV-6 [64, 203]. Intrathecal anti-HHV-6 IgG antibodies were detected in around 20% of MS patients [203]. In the periphery HHV-6 mRNA and DNA have been found more frequently in PBMC and serum, respectively, in MS patients than in controls [204]; and a significantly increased frequency of MS patients with active HHV-6 infection were in relapse than in remission [182, 205, 206]. Serum IgM antibodies are detectable in the early events of an infection and an increased frequency of anti-HHV-6 IgM antibodies have been detected in early stages of MS [191, 207] indicating a role for HHV-6 in disease onset and periods of active MS disease. An increased frequency and titers of serum IgG antibodies against the viral latency associated protein U94 has also been detected [208]. This is consistent with association studies of MS and EBV where a slight increase in IgG titers against the latency associated protein EBNA-1 is frequently seen [209-211]. Furthermore, increased titers of serum IgG antibodies against HHV-6 were shown to positively associate with relapse risk in RRMS [212]. This association was seen after correction for IgG titers against the EBV proteins EBNA-1 and VCA. It is tempting to conclude that this supports the notion of an increased frequency of HHV-6 reactivation as a mechanism of disease activity. However, it cannot be ruled out that this rather reflect a locally increased immune activity in the CNS, which triggers viral reactivation, given that the CNS is a site of latency for HHV-6.

In an experimental animal study marmosets were challenged intravenously (i.v.) and HHV-6A but not with HHV-6B gave clinical MS like symptoms and lesions seen by MRI [213]. Additional studies support the notion that HHV-6A is more central in MS. Firstly, HHV-6A seems to be more neurotropic, and moreover, an increased cellular immune response against HHV-6A, but not HHV-6B, has been reported in MS patients compared to healthy controls [214]. In the marmoset study the animals to be exposed to HHV-6A where divided into two groups based on infection route, i.v. or intranasal (i.n.). Whereas the i.v. challenged animals exhibited clinical symptoms and development an antibody response the i.n. challenged animals did not. This suggested that the underlying pathogenesis may lie in the immune response rather than the primary infection itself. A more prominent role for HHV-6A in MS is further supported by data from another animal model where glial cells from transgenic mice expressing human CD46 supported HHV-6A but not HHV-6B replication. Upon *in vivo* infection HHV-6A DNA could be detected in the brains of these mice for up to 9 months (Reynaud et al., 8th International Conference on HHV-6&7, April 2013). In humans a predominant role for HHV-6A in MS is supported by the increased *in vivo* and *in vitro* neurotropism, an increased seroreactivity to HHV-6A compared to HHV-6B infected cells [201] and increased detection of HHV-6A DNA in MS serum and CSF compared to HHV-6B [215].

Even though a majority of the studies show a positive association between HHV-6 and MS there are a number of conflicting reports where no association could be shown. An obstacle when reviewing the literature on HHV-6 in MS is the broad variety in the techniques used. A careful meta-analysis on the literature published between the years 1966 and 2009 was performed assessing the quality of the studies and their results [216]. Here, 60% of studies that were top ranked regarding study design, according a set of pre-determined criteria [217], showed significant differences between MS patients and controls. To conclude, in MS HHV-6 seems to be important in early stages of the disease and to have a direct effect on the CNS. Furthermore HHV-6A seems to have a more prominent role than HHV-6B. Even though most studies show a positive association the concept is still highly controversial and additional and carefully performed studies are urgently needed.

1.3.4.1 Clinical trial

As for the EBV field, the idea that a clinical trial where one could observe a clinical improvement in patients with neuroinflammatory disease after antiviral treatment targeting HHV-6 would be a proof of principle is strong in the HHV-6 field as well. This approach was actually taken in 2005 where MS patients were treated with valaciclovir for two years [218]. Trends were seen in patients with severe MS (expanded disability status scale (EDSS) >4) for treatment effects on clinical measures such as time to first relapse after trial onset but the differences to the placebo group was not statistically significant and not supported by MRI measures. No effects were seen for patients with mild MS (EDSS ≤4). For the virological outcome anti-HHV-6 IgM antibodies were measured and no effect was seen in any of the groups tested. Valaciclovir is a prodrug for aciclovir that is taken orally and an *in vitro* efficacy against HHV-6 had been shown a few years prior to the trial [219]. Furthermore it had been shown to access the CNS at concentrations of up to 22% of the plasma level [220]. Given the indications of a direct role for HHV-6 in the CNS this is an important feature. The choice of drug seemed vice until it a few months later was shown that it had no *in vivo* effect on HHV-6 [221]. Therefore, a first step in another attempt of this approach again would be to choose a drug with approved *in vivo* efficacy.

Chronic fatigue syndrome (CFS) is another condition where the patient is suffering of excessive enervation and display activation of the immune system. Some studies suggests an association between CFS and HHV-6, but as for HHV-6 and MS, the hypothesis is controversial as other studies show conflicting data [222, 223]. Ganciclovir is a drug originally developed against CMV that also has well documented *in vitro* and *in vivo* efficacy against HHV-6 [224]. Valganciclovir (VGCV) is an oral prodrug for ganciclovir [225] that showed promising result in a small study where CFS patients positive for IgG against EBV and HHV-6 were treated [226]. They improved clinically and also showed decreased IgG titers against both viruses. This suggested that HHV-6 is important in CFS and that anti-HHV-6 IgG antibodies are a good measure of viral outcome but a more controlled study was needed to convince people (7^{th} International conference on HHV-6 & 7). In a subsequent randomized clinical trial

by the same research group [227] CFS patients were treated again with VGCV. Statistically significant beneficial effects on clinical measures in a subset of patients were observed but no decrease in IgG antibodies against neither HHV-6 nor EBV could be detected. This example underlines the importance of using adequate virological measures to identify the treatment effect on the virus. Because, even though the patients did improve clinically, the annoying fact that the anti-HHV-6 IgG titers did not drop are in favor for the idea against a HHV-6-causation. Off course one could argue that if a treatment improves the clinical condition it warrants further use regardless of if the mechanisms behind are known, as for treatments with IFN-β and GA in MS. But if the ultimate aim, in the effort to link HHV-6 with a disease such as CFS or MS, is to establish anti-HHV-6 treatment as a first line intervention in the clinic or even vaccination of healthy individuals, distinct virological measures are vital. Hence, if HHV-6 were to be proven to cause MS, what mechanisms might underlie the initial events?

1.3.4.2 Hypothesized mechanisms for HHV-6 induced autoimmunity

As discussed in section 1.2.2 DC are the primary conductor of T cells. DC mature upon exposure of PAMPs and thereafter can activate T cells. As PAMPs are not present in self-tissue it seems likely that they should have to be present in breakage of tolerance and autoimmunity. This was suggested by Polly Matzinger who proposed a model where APCs need a danger signal in combination with an antigen to elicit an immune response [228]. An example of this idea in practice is given by the animal model for MS, EAE. It has striking similarities with MS with infiltration of lymphocytes in the CNS and demyelination [229]. An important difference is however, that whereas the MS etiology is unknown the onset of EAE can be controlled. Typically myelin proteins are injected together with adjuvants to trigger autoimmune responses against myelin proteins. The task of the myelin proteins is to provide a myelin specific response. However, as exemplified by the GA treatment administration of the self-antigen alone is not sufficient to induce breakage of tolerance. Therefore the task of the adjuvant is to provide the immune system with a danger signal to triggering an immune response. Thus, if EAE is an adequate model of MS, these two components, specificity and danger signals have to be included in a model of MS etiology.

1.3.4.2.1 Molecular mimicry

The two criteria danger signal and specificity are fulfilled in the molecular mimicry hypothesis. This hypothesis propose that this might be elicited with infection with a pathogen that have a peptide that can bind to the HLA molecule in a way that makes the peptide-HLA complex structurally similar at the site for TCR binding compared to myelin peptide binding [9]. Support for this model is given by findings of a seven-mere peptide from the HHV-6 protein U24 (residues 4-10) that is identical to a peptide in the myelin basic protein (MBP) (residues 96-102). These peptides were extended by the flanking three residues in each direction and the MBP peptide were used to expand MBP specific T cells. When the HHV-6 U24 peptide were used to re-stimulate these cells they were further expanded suggesting that the viral and MBP peptides can cross

react [230]. Molecular mimicry has been seen also between MBP and EBV in MS [231].

1.3.4.2.2 Host cell protein incorporation

We have had another working hypothesis on HHV-6 induced autoimmunity based on findings that HHV-6 can include host cell proteins such as CD46 and p53 in its virion [232, 233]. This seems to be a common phenomenon for enveloped viruses and has been shown for sever other viruses such as HTLV-1, Vaccinia Virus, and HIV-1 [234-238]. Several proteins involved in immune regulation are known to be incorporated into the HIV virion and the virus can in itself thereby acquire antigen presenting and activating functions, which benefits the virus with increasing replication efficacy [239]. An enveloped virus (Vesicular stomatitis virus, VSV) has been suggested to selectively incorporate host cell surface proteins into budding virions [240] and furthermore to be an highly efficient trigger of autoreactive T cells from patients with postinfectious encephalomyelitis syndrome, when grown in myelin protein-expressing cell cultures [241]. This mechanism of virus-induced autoimmunity has also been suggested to occur in graft versus host disease, where the enveloped virus CMV incorporates a host cell protein (CD13) during replication, leading to the induction of autoimmunity against this protein [242, 243]. For MS, such a mechanism requires a virus that replicates in the myelin-producing oligodendrocytes.

The candidate virus was found in HHV-6 since it, as described above, can infect oligodendrocytes and since it can establish latent infections with reactivations that coincide with the relapsing-remitting pattern seen in most MS patients. Furthermore, a number of studies suggest an association between HHV-6 and MS. Thus, our working hypothesis has been that breakage of tolerance and MS onset is induced when: Firstly, HHV-6 replicates in oligodendrocytes and incorporate myelin proteins into its virion. Secondly, an APC in the periphery engulf the myelin protein containing virion and is activated via ligation of viral motifs to PAMPs and TLRs. Thirdly, that anti-myelin specific T cells can be activated by these APCs as the engulfed HHV-6 virion carried myelin proteins. Lastly, these activated myelin specific T cells can enter the brain and, if they express CD8, destroy oligodendrocytes or, if the express CD4, constitute help to B cells which start to produce anti-myelin antibodies that tag myelin proteins for macrophage destruction (figure 5).

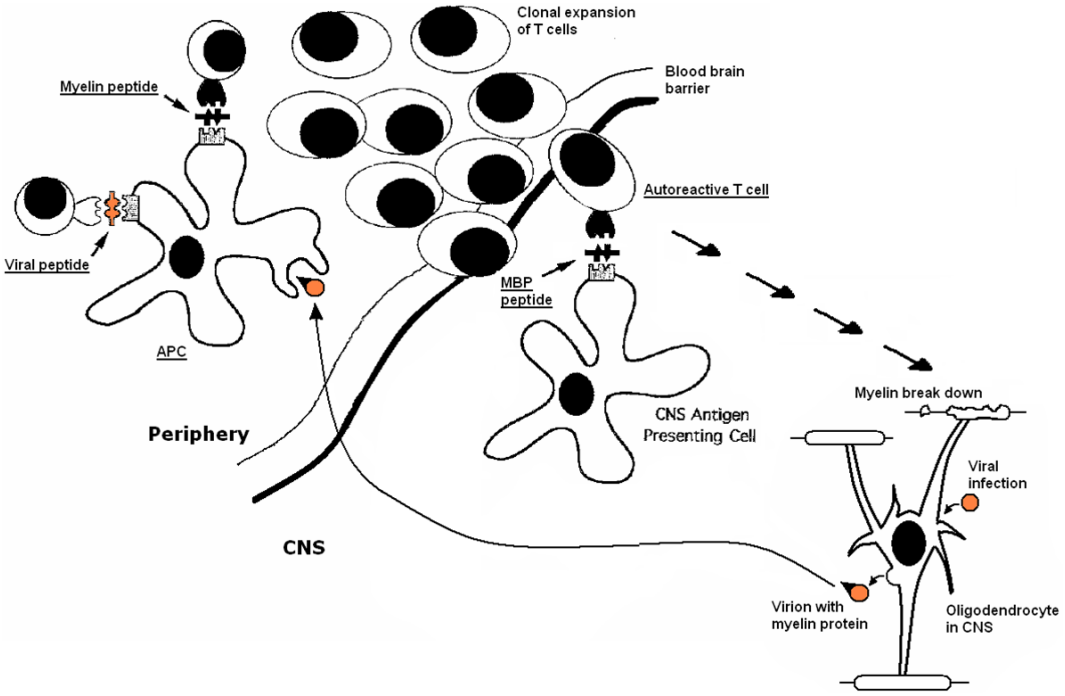

Figure 5. Schematic view of the hypothesis of tolerance breakage via host cell protein incorporation into virus particles. The event starts with a neurotropic virus, such as HHV-6A, infecting oligodendrocytes. When it has budded off the virus capsid is wrapped in host cell membrane that contains host cell surface proteins, such as myelin proteins.

2 AIMS OF THE THESIS

The overall aim of this thesis was to investigate the role of HHV-6 in MS. More specifically the individual projects were aimed:

1. To develop a robust method for HHV-6 titer assessment that is easy to interpret.

2. To assess the potential of HHV-6A as an adjuvant, for triggering DC activation and T cell proliferation induction.

3. To assess the frequency of active HHV-6 infections in MS patients compared to healthy controls, as seen by viral DNA in serum.

4. To assess if the serological IgG antibody response against HHV-6 differs between MS patients and healthy controls, and furthermore if factors previously associated with MS susceptibility are associated with the antiviral IgG response.

5. To assess if host genetics have an effect on the serological IgG antibody response against HHV-6.

3 MATERIALS AND METHODS

3.1 EXPERIMENTAL STUDIES

3.1.1 Cell culture and HHV-6A propagation

In studies I and II cells of the T cell line HSB-2 were cultured and expanded in GlutaMAX containing RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 mg/ml streptomycin, referred to as complete RPMI. In paper II monocytes were isolated from buffy coats purchased from the Blood Transfusion Clinic at the Karolinska University Hospital. Briefly, monocytes were retrieved from the buffy coats using commercial kits and density gradient centrifugation on lymphoprep. For the generation of DC, purified monocytes were cultured for seven days in complete RPMI supplemented with 6.5 ng/ml of recombinant human IL-4 and 250 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), fresh cytokines were added on day three. The yield of DC was determined by FACS and was typically >70% of CD14-CD1a+ live cells.

HHV-6A strain GS [1] was propagated in the T-cell line HSB-2. When the cytopathic effect (CPE) was >50% the cell culture was centrifuged for 10 min at 300 g and the supernatant was harvested and stored in aliquots at -80°C. The 50% tissue culture infective dose ($TCID_{50}$) was determined by ocular inspection of the cytopathic effect or a Q-PCR based read-out as described in paper I. For the infection experiments in paper I and II HSB-2 cells or DC were inoculated with 10^{-1}-10^{-6} multiplicity of infection (MOI) for three hours before they were washed with incomplete RPMI medium and further cultured in complete RPMI. For the DC cultures the complete RPMI was supplemented with IL-4 and GM-CSF. As control for non-replicating infection, HHV-6A was inactivated with UV irradiation for 20 minutes. In paper I we saw by $TCID_{50}$ that this treatment completely eliminates the replication capacity. After inoculation the infection was followed by longitudinal sampling for up to fourteen days and analyzed by Q-PCR targeting an immediate-early gene as described previously [244]. DNA was extracted using a commercial bead based kit that allowed for simultaneous extraction of up to 96 samples. The infected cells were also analyzed by immunofluorescence assay (IFA) by fixation onto glass slides with a 1:1 mixture of acetone and methanol at -20°C for 10 minutes, for blocking 5% goat serum and 3% bovine serum albumin in PBS was used, and the cells were stained with primary mouse MAbs specific to the late HHV-6 glycoprotein gp116/54/64 or early protein p41. The staining was visualized by an Alexa 633 conjugated F(ab')2 fragment of goat anti-mouse IgG (Invitrogen). Staining with 4',6-diamidino-2-phenylindole (DAPI) was used to visualize the cell nuclei.

3.1.2 $TCID_{50}$ set up

For the $TCID_{50}$ culture plate set up, cell suspensions of 40 µl containing 10^4 HSB-2 cells per well were seeded in round bottom 96-well culture plates. The cells were inoculated for three to four hours with 160 µl of five-fold dilutions of the HHV-6A

batch to be tested in six replicates per dilution. As negative controls for replication, mock and medium controls were included in triplicates. Mock was supernatant from uninfected HSB-2 cells. The culture plate was shaken every 20-30 minutes to enhance binding of virions to the cell surfaces. The cells were washed once before 50-70 µl of cell suspension were sampled from every well and stored in -80°C as zero days post infection (dpi) samples. The cells were allowed to sediment to the bottom of the wells in the washing step. After sampling the remaining cell suspensions were further incubated for seven days. At seven dpi, the zero dpi culture plate was thawed and subjected to DNA extraction, using the bead based kit, and Q-PCR for viral DNA measurement as described in section 3.1.2.

3.1.3 Assessments of DC functions

As positive control for maturation in paper II, DC were cultured with 100 ng/ml lipopolysaccharide (LPS) together with 500 U/ml IFN-γ. As negative control DC were inoculated with supernatant from uninfected HSB-2 cells, referred to as mock.

3.1.3.1 Flow cytometry

For flow cytometry in paper II, HHV-6A or mock inoculated DC were labeled with MAbs specific for the following markers: CD83, HLA-ABC, CD14, HLA-DR, CD40, CD86 and CD1a. To visualize and exclude dead cells from the analysis, the cells were also incubated with a live/dead marker. All labeling were performed on ice for 30 min in PBS containing 2% FCS, 5 mM EDTA and 0.01% sodium azide (NaN_3). All flow cytometric analyses were performed with a CyAn flowcytometer and data was analyzed using the software FlowJo. For detailed information on the fluorochrome conjugation to each MAb see the materials and methods section in paper II enclosed in the end of this thesis.

3.1.3.2 Cytokine measurements

In paper II, supernatants from HHV-6A infected and control DC were examined for various cytokines important in inflammation and DC maturation. These included type I, II and III IFNs, IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70. Myxovirus resistance protein A (MxA) gene expression is only induced by type I and III IFNs, and hence represent a sensitive indicator of total levels of bioactive type I and III IFNs in samples [245, 246]. For analyses of the total level of bioactive type I and III IFNs we therefore used an MxA gene expression assay [247]. Briefly, levels of MxA mRNA were measured in cells of the lung cancer cell line A549, exposed to 100 µl supernatants collected from the DC cultures at 3 dpi. As standard we used therapeutic IFN-β. The A549 cells were exposed to the samples for 6.5 h followed by lysis in lysis buffer containing 50% Nucleic Acid Purification Lysis Solution in 1xPBS. The culture plate with cell lysate was stored in -80°C over night before mRNA was extracted. Thereafter, the mRNA was converted to cDNA using the High Capacity cDNA Transcription kit, prior to Q-PCR analysis with primers and probes targeting the MxA gene and 18S gene, where 18S was used as endogenous control of mRNA expression. To further determine the specific types of type I and III IFNs produced by

DC IFN-α, -β and -λ specific enzyme linked immunosorbent assays (ELISAs) were run using in-house methods for IFN- α and -λ and a commercial kit for IFN-β. For measurements of IL-8, IL-1β, IL-6, IL-10, TNF and IL-12p70 cytometric bead arrays (CBA) were used.

3.1.3.3 Mixed lymphocyte reaction

In paper II, the allostimulatory capacity of HHV-6A inoculated DC was evaluated by mixed lymphocyte reaction (MLR). Here 300–3000 live DC were co-cultured with 10^5 allogenic live T cells per well. Determination of cell numbers was performed using a phase contrast microscope after Trypan blue staining. CD3+ CD8+ and CD3+ CD4+ T cells were isolated from PBMC from buffy coats and purified as described above with the use of magnetic micro bead kits, according to manufacturer's instructions. MLRs were incubated for a total of 96 hours. Eight hours before termination, 1 mCi [3H]-thymidine per well was added. The culture medium was complete RPMI supplemented with 50 mM 2-ME. After being pulsed with [3H]-thymidine, the cells were harvested and the degree of [3H]-thymidine incorporation in proliferating T cells was analyzed. For 3H-thymidine incorporation and harvesting of cells the following material was used: 3H-thymidine, Tomtec harvesting machine, glass fiber filter, melt-on scintillation sheet, sample bags and 1450 Microbeta counter. To determine if HHV-6A infection of DC can skew the T helper cell profile into Th1 or Th2 lineages CBA was performed on MLR supernatants targeting the cytokines IL-2, IL-4, IL-6, IL-10, TNF and IFN-γ.

3.2 PATIENT STUDIES

3.2.1 Patients and samples

In paper III, stored plasma, serum and CSF samples taken as part of the clinical practice between the years 2002-2005 of patients with MS, Guillain-Barré syndrome (GBS), chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) and headache were used. The headache patients were used as control group. The MS patients were diagnosed using the McDonald criteria [6], the GBS patients using the Asbury criteria [248] and the CIDP patients using criteria from the American Academy of Neurology AIDS Task Force [249]. The MS patients were divided into three subgroups; treatment naïve, neutralizing antibody negative (NAb-) IFN-β treated and NAb+ IFN-β treated. The treatment naïve patients were newly diagnosed or showed signs of MS but had not yet fulfilled the full diagnosis criteria for MS. Therefore they are referred to as possible MS.

In papers IV and V plasma samples were used from patients with MS, diagnosed using the updated McDonald criteria [7], and from healthy controls collected between the years 2005 and 2009 for research use.

3.2.1.1 DNA extraction and nested PCR

In paper III, DNA was extracted from plasma or serum and cerebrospinal fluid (CSF) from patients with possible MS, GBS and CIDP using a commercial kit. For

contamination control water was added to every fifth column. DNA was eluted in buffer and stored at -80°C. Subsequently, the eluates of all samples were screened for HHV-6 DNA using a nested PCR targeting the major capsid protein (MCP) gene as previously described [158]. Tests were considered valid if all water controls remained negative and if the viral laboratory strains U1102 and Z29, used as positive controls, were amplified. The MCP nested PCR sensitivity was 240 copies/ml, as determined by Q-PCR as previously described [244].

3.2.1.2 Anti-HHV-6 IgG ELISA

In papers IV and V, plasma anti-HHV-6 IgG antibody titers was assessed using a commercial ELISA Kit, with HHV-6B Z29 strain viral lysate as antigen, according to the manufacturers' protocol. Briefly, all plasma samples (n=933) were run in at least duplicate wells. The titer results are given as optical density (OD) ratios, calculated by the OD value for the particular sample divided by two times the OD value for the negative control. The results were interpreted as suggested in the manufacturers' protocol; OD ratios ≤0.75 were regarded as negative and OD ratios ≥1.00 as positive. Borderline samples (OD ratio 0.76-0.99, n=36) were excluded from the antibody status analysis but included when titers were analyzed. Samples with an OD ratio ≥0.75 were accepted only if the OD values had a coefficient of variance (CV) of less than 25%. The OD ratio of the positive controls on all plates had an inter-assay CV of 13%.

In paper IV, plasma IgG responses against the early HHV-6 protein p41/38 and total IgG was also investigated in a subset of 67 patients and 67 healthy controls using commercial ELISA kits. All samples were run in duplicates and the experimental procedures were performed according to the kit specific protocols. For plasma IgG responses against p41/38 nOD values were obtained as for the anti-HHV-6 responses described above. For total IgG absolute quantification using a standard curve was performed.

3.2.1.3 HLA typing and measurement of vitamin D level

All samples in paper IV were genotyped typed for HLA-A and HLA-DRB1 using a commercial kit based on sequence-specific primers [250]. Genotypes for HLA-A and DR were unavailable for 17 and 23 patients, respectively. For these, HLA allele status was imputed using HLA*IMP01 [251] from high density genotypes generated in a genome-wide scan for MS susceptibility genes [12]. Levels of 25-hydroxyvitamin D_2 and D_3 were measured in the plasma samples from all subjects in paper IV as previously described [252].

3.2.2 Genome-wide SNP typing and gene identification

In paper V, genome-wide single nucleotide polymorphism (SNP) markers were genotyped as part of the International Multiple Sclerosis Genetic Consortium (IMSGC) Welcome Trust Case Control Consortium 2 (WTCCC2) MS Genome wide association study (GWAS) project using the Illumina Human660-Quad chip (Illumina, San Diego, CA, USA). Genotype calling and SNP markers were quality controlled as previously

described [12]. After the quality checks 483 232 markers remained, and of these 472 716 markers were autosomal.

Using the free and web based HapMap dsSNP tool accessible via the NCBI webpage (www.ncbi.nlm.nih.gov/projects/SNP/) genes within 50kb of each SNP were identified. As GWAS approaches yields very large amounts of data. Therefore this rather strict and well established threshold [11] was chosen, together with the decision to investigate only the nearest gene to each SNP, to limit the amount of candidate genes. If a gene laid within 50kb to the SNP together with a miscellaneous RNA (miscRNA), non-coding RNA or a pseudogene, the gene was listed. The rational for this was that the functional properties of miscRNAs are largely unknown and that non-coding RNAs and pseudogenes have lost the protein coding ability.

3.3 STATISTICAL ANALYSES

In paper I, Excel was used to calculate coefficients of variance (CV) when comparing the robustness different titration methods. In paper II Student's t-test and one-way or two-way analysis of variance (ANOVA) with Bonferroni's correction for multiple testing were applied using GraphPad. In paper III Fisher's exact tests were performed using GraphPad. In paper IV linear regression analysis was performed using R version 2.15.2. All samples (n=933) were analyzed together to assess relationships between anti-HHV-6 IgG nOD levels and the independent variables gender, affection status (MS patient or healthy control), smoking (never smoker or past/present smoker at sampling), age at sampling, vitamin D levels and *HLA-A*02* and *HLA-DRB1*15* carriership, and if statistically significant relationships existed between these variables. Multiple linear regression models were constructed for MS cases and controls together to determine if these variables interacted. For the MS case only model, the variables disease duration, treatment or no treatment and specific treatment types were included. Mann Whitney U tests were employed in STATISTICA when comparisons between two groups were made. For the MS patient group, Kruskal-Wallis test was performed in STATISTICA to determine if the nOD levels differed between the groups: treatment naïve (n=139), IFNβ (n=41) and Glatiramer acetate (n=11).

In paper V logistic and linear regression analysis were performed to assess the effects of host genes, via GWAS SNP markers, on anti-HHV-6 IgG antibody status and levels. SNPs with high linkage disequilibrium to other SNPs in the analysis ($r2<0.2$) were first removed using PLINK 1.07 [19]. Subsequent analyses were performed on the MS patients and healthy control groups assembled, as no differences in anti-HHV-6 IgG status or nOD levels was observed between the groups. Human leucocyte antigen HLA allele status were assessed by imputing the HLA alleles using HLA*IMP01 [21] from SNPs within the HLA region. HLA association was tested to both HHV-6 IgG status, using logistic regression, and to normalized HHV-6 levels using linear regression. Linear regression analysis for the GWAS analysis was performed in two ways; with all HLA alleles associated to HHV-6 levels included as covariate, respectively not included as covariates. Threshold for suggestive association was set to $p<1*10^{-4}$ as in previous studies [11].

4 RESULTS AND DISCUSSION

4.1 PAPER I

It is crucial to have control of the viral titer in experimental work with viruses. In paper I [253] we developed and validated an alternative read-out approach for the 50% tissue culture infectivity dose ($TCID_{50}$) method [194] for HHV-6. The experimental procedure is described in more detail in paper I of this thesis. Briefly, we measured the increase in viral DNA load of every titration well in $TCID_{50}$ culture plates using real time quantitative polymerase chain reaction (Q-PCR) after seven days of culture. The established method of ocular inspection for CPE is problematic at the borderline of infection in titration series' as the cells enlarged due to infection tend to enlarge less with increased dilution of the virus (figure 6). IFA for $TCID_{50}$ read-out [195] or for infectious units [196] is more distinct but the monitoring of individual cells is laborious. In addition, monitoring of individual cells for CPE or IFA implies a risk to misinterpret the cell's positivity.

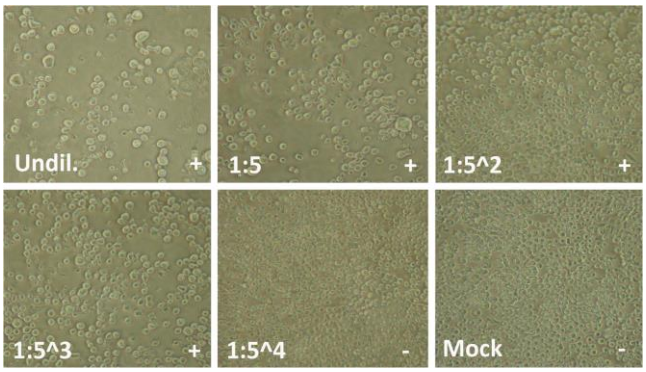

Figure 6. Example of ocular inspection of HHV-6A (GS strain) infected HSB-2 cells. Dilutions and positive (+) and negative (-) results in Q-PCR $TCID_{50}$ assessments are indicated. Undil: undiluted virus supernatant, 1:5; five times dilutions of virus supernatant etcetera. Photographs are taken using a lens for ten times enlargement.

4.1.1 Assay development

Primarily, the harvest time-point had to be determined. Longitudinal sampling of HHV-6A inoculated cell cultures was performed for ten days. A plateau was seen at seven days post infection and was therefore chosen as the harvest time point. Secondly, the cut-point for viral DNA load increase that corresponded to true infection had to be set. To do this, cells from every well in three different $TCID_{50}$ culture plates were subjected to IFA and the results were compared to the Q-PCR results of every culture well. The optimal cut-point was found at ten times increase in viral DNA load that corresponded to viral protein expression in 93% of the wells (figure 7).

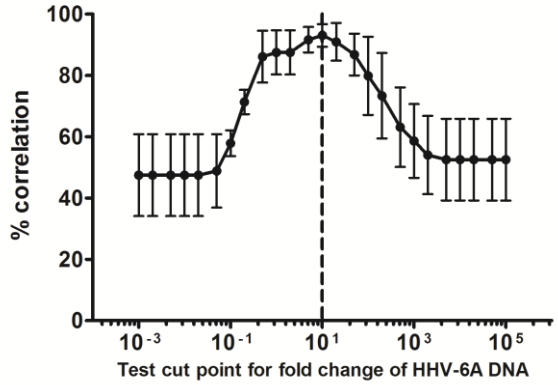

Figure 7. The optimal cut-point for infection was determined to a ten times increase in viral DNA load. At this point the correlation to viral protein expression was seen in 93% of the wells using IFA targeting the late viral protein gp116/54/64. Data shown are mean results (± SEM) for three $TCID_{50}$ plates.

4.1.2 Assay validation

Validation is a vital step when developing a new method. To validate our method we tested four different batches of HHV-6A (GS strain) multiple times and compared the titer results with the results from ocular inspection and IFA read outs of $TCID_{50}$. In addition the use of IFA to determine the number of infectious units was used. For all batches the inter-assay CV values were measured. In this setting the inter-assay CV value gives a measure on the variability in the results when the assay is performed several times on the same virus batch. For our method the inter-assay CV was 73% whereas it was 66%, 25% and 77% for the ocular inspection read-out for $TCID_{50}$, IFA read-out for $TCID_{50}$ and infectious units respectively. These numbers are very high. It is not surprising however as they are all biological assays that depend on many factors such as the status of the cells, which in turn can be influenced by many factors such as the freeze thawing procedure. The efficacy of the infection is also a critical parameter, which in turn can depend on various factors such as the number of times the culture plate is shaken during incubation. Therefore, a more accurate measure on the robustness of a bioassay is the intra-assay CV value. In this setting the intra-assay CV value gives a figure of the robustness of the reading at an individual performance of the assay. To determine the intra-assay CV for our method we performed parallel duplicate extractions and Q-PCRs for three $TCID_{50}$ culture plates to see to what extent the extraction and the Q-PCR influenced the results. For ocular inspection the intra-assay CV value was determined by twelve $TCID_{50}$ culture plates read by two persons independent of each other to assess the extent of individual interpretations of infection. For the IFA read-out of $TCID_{50}$ the intra-assay CV value was determined by duplicate staining and counting of cells from all wells from two different culture plates. Finally, for the infectious units approach the intra-assay CV value was determined by four parallel staining procedures of cells from one run. The intra-assay CV value for our method was 9% compared to 45%, 14% and 43% for the ocular inspection read-out of $TCID_{50}$, the IFA read-out of $TCID_{50}$ and infectious units approach respectively. Comparing the intra-assay CV values gives the conclusion that our method was more robust than the established methods.

Some virologists use the HHV-6 DNA copy numbers in the virus batch as a rough estimate of the amount of infectious viral particles. We tested this idea and the viral DNA load did not correlate to the viral titers, when determined using any of the three established methods, nor when determined using our method. This indicates that measuring viral DNA in batch's supernatants is insufficient to correctly assign the infectivity of a batch. Instead biological assays should be performed.

4.1.3 Conclusions

Even though our novel read-out method proved most robust and is easier to interpret it has limitations. One is encountered when large amounts of free viral DNA is present. This DNA is unable to reach the cells' interior and get replicated and furthermore yields a high signal in the sample before incubation. This can then mask the signal of the replicated DNA at seven days post inoculation giving the impression that a low amount of viral DNA has actually been replicated. This problem is typically seen in the culture plate wells that have been incubated with undiluted or five times diluted virus supernatants. A manifestation of this problem is when the viral DNA increase does not reach the cut-point in undiluted or five times diluted wells, but further down in the dilution series. In this scenario, the results of the wells further up in the dilution series can be excluded.

To conclude, it is important that standardized and validated titration methods are harmonized within the HHV-6 field. This would facilitate comparisons between experimental studies performed in different labs. An assay that is easy to perform and interpret seems preferable, especially for people new in the HHV-6 field.

4.2 PAPER II

The incorporation and molecular mimicry hypotheses are based on the idea that self-proteins, such as myelin proteins, or viral proteins that mimic myelin proteins, become immunogenic when they are taken up by APCs together with HHV-6 virions. A first step to investigate this hypothesis is to assess if HHV-6 can mount an adjuvant effect on APCs in the absence of self-proteins. As DC are the most potent APCs that are central for activation of CD4+ and CD8+ T cells [102], this project was focused on HHV-6A exposure of DC [175].

4.2.1 HHV-6A cannot replicate in DC

Previous studies report contradictory results on the replication capacity of HHV-6A in DC [143, 173]. Whereas Hirata *et al.* [143] suggests that DC can support HHV-6A (U1102 strain) replication, Smith *et al.* [173] suggests that DC cannot support HHV-6A (GS strain). Therefore we wanted to further investigate this notion. We did not see any increase in neither viral protein nor DNA (figure 8) supporting the report by Smith *et al*. The discordant results might be due to the different viral strains used or different passages of the virus, as the characteristics of cells and viruses are altered upon *in vitro* cultivation [151, 156]. To control for degraded viral batches or suboptimal infection procedures susceptible cells were infected in parallel with DC using the same virus supernatants. Both we and Smith *et al.* did then see infection in the susceptible cells suggesting that lack of viral replication seen in DC was not due to problems with the virus stock or infection procedure.

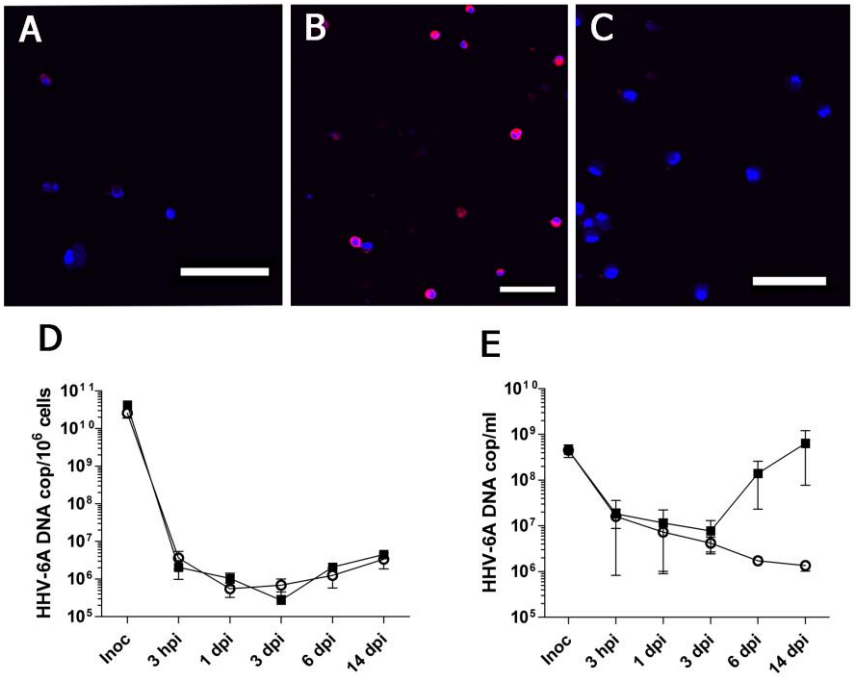

Figure 8. HHV-6A infection for 6 to 7 days of DC (A) or HSB-2 cells (B), as positive control. As negative control DC were inoculated for 6 days with mock, supernatant from uninfected HSB-2 cells (C). The cells were stained with an anti-HHV-6 MAb (red) specific for the late viral protein gp116/54/64 and with DAPI (blue). Scale bars are 50µm. Replication was also assessed by Q-PCR analysis of intracellular (D) and extracellular (E) viral DNA after HHV-6A inoculation of DC (open circles) and HSB-2 cells (filled squares). Inoc: inoculum, dpi: days post infection, hpi: hours post infection. Data are means (± SEM) for three donors.

4.2.2 HLA-ABC up-regulation via IFN-α

To assess if DC are matured upon HHV-6A inoculation we analyzed the supernatants for type I IFN, and the cells for surface expression of various activation markers. We saw that exposure to HHV-6A induced up-regulation of HLA-ABC, HLA-DR and CD86 on the DC cell surface (figure 9A). Furthermore, our data show that exogenously added IFN-α can mediate up-regulation of HLA-ABC (figure 9B) as previously reported [77], and that DC can produce IFN-α upon HHV-6A exposure (figure 9C). The link between HHV-6A induced IFN-α secretion and HLA-ABC surface expression is further supported by our finding that addition of an anti-IFN-α antibody in the culture medium prevent the up-regulation of HLA-ABC (figure 9D).

Interestingly, when the virus inoculum had been UV-treated prior to infection, DC exhibited a trend of reduced IFN-α response, compared to when the virus inoculum had not been UV-treated (figure 9B). This suggests that the UV-treatment might destroy important motifs of the virion that can induce IFN-α secretion, such as TLR ligands. Another option is that HHV-6A do replicate at low levels, under the detection limit of the IFA and Q-PCR assays used, resulting in an elevated number of ligands accessible for host cell receptors such as TLRs. As discussed in section 1.1.4 type I IFNs, such as IFN-α, are central molecules in antiviral immunity and important to measure when assessing the effect of a virus on DC functions.

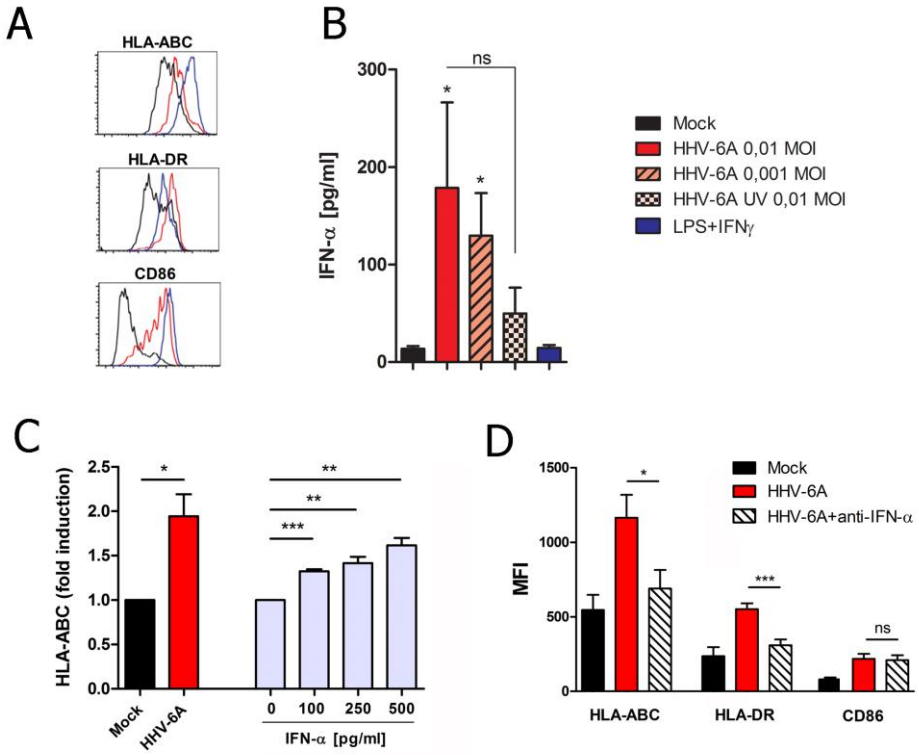

Figure 9. DC respond to HHV-6A infection at 3 days post infection (dpi) with increased surface expression of HLA-ABC, HLA-DR and CD86 compared to mock, as seen with flow cytometry (A). DC produce IFN-α at 3 dpi upon HHV-6A as seen with ELISA (B). HLA-ABC surface expression is up-regulated at 1 dpi upon HHV-6A exposure and also upon addition of exogenous IFN-α for 24h (C). In another experiment DC were inoculated with HHV-6A for three hours before they were washed and cultured in medium in the presence or absence of a polyclonal anti-IFN-α antibody in the culture medium. At 3 dpi the cells were harvested and analyzed for surface expression of HLA-ABC, HLA-DR and CD86.

Figure 9 (continued). Representative histograms from live CD1a+ DC from one of seven donors is shown in panel A. Data is shown as mean results (± SEM) for at least six donors and analyzed using ANOVA with Bonferroni's multiple comparison test for panel B and for four donors and analyzed using Student's t-test for panels C and D. *p<0.05, **p<0.01, ***p<0.001. DC where inoculated with HHV-6A supernatants at 0.01 MOI for all panels.

Previous studies assessing cell surface HLA-ABC expression on DC after HHV-6A exposure report conflicting results. Whereas Hirata *et al.* [143] reported a down-regulation Smith *et al.* [173] reported an unaltered HLA class I expression. This indicates that down-regulation is dependent on productive viral replication given that Hirata *et al.* saw viral replication, whereas we and Smith *et al.* did not, as discussed above.

4.2.3 Modulation of inflammatory cytokine secretion

Inoculation with HHV-6A reduced the capacity of DC to secrete IL-8 compared to mock (figure 10A). When the virus had been UV-treated prior to inoculation this effect was lost (figure 10B), again suggesting that HHV-6A might replicate at low levels and that the reduced secretion of IL-8 is dependent on replication capable virus. If HHV-6A has an ability to attenuate the IL-8 secretion *in vivo* then attraction of neutrophils might be hampered, as IL-8 can induce trafficking of neutrophils across vascular walls [254]. Since neutrophils are important cells of the innate immune system [255], impaired IL-8 secretion might constitute a potential immune evasion strategy by the virus. However, this effect seems to be overridden when other inflammatory stimuli are present, as addition of LPS and IFN-γ in the culture medium led to slightly elevated IL-8 secretion by HHV-6A exposed DC compared to mock, although not significantly (figure 10C).

HHV-6A exposure also led to augmented TNF and IL-12p70 levels (figure 10D and 10E). This is in contrast to two studies by Smith *et al.* which suggest that pre-exposure with HHV-6A suppresses LPS and IFN-γ induced IL-12p70 secretion by DC [173] and also by macrophages [172]. This divergence might be due to different procedures used for virus propagation. We used the virus supernatants directly after centrifugation of the virus cultures, whereas Smith *et al.* ultracentrifuged their supernatants prior to inoculation. In their discussion they argue, that ultracentrifugation should be performed in order to remove cytokines from the supernatant that might affect the IL-12p70 secretion. To investigate this notion we tested our virus inoculum and mock with CBA targeting IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, TNF and IFN-γ, and the *MxA* bioassay targeting type I/III IFN. All cytokines were present at negligible levels close to the lower detection limits of the assays and no differences were detected between virus inoculum and mock (data not shown). Therefore, we conclude that the differences are likely not due to the presence of other cytokines. Another option is that cell debris from dead cells, used for propagation, is present in the crude supernatant and are removed upon ultracentrifugation. One could argue that ultracentrifuged virus is cleaner than crude virus supernatants but the question is what preparation that best mimics the *in vivo* situation, clean virions or a virion and cell debris mixture? Anyhow, the idea that cell debris affects the results could be tested by redoing the experiments with ultracentrifuged and non-ultracentrifuged virus supernatants in parallel and also

treat DC with lysed uninfected cells to assess if the effect is virus specific, cell debris specific or a combination of both.

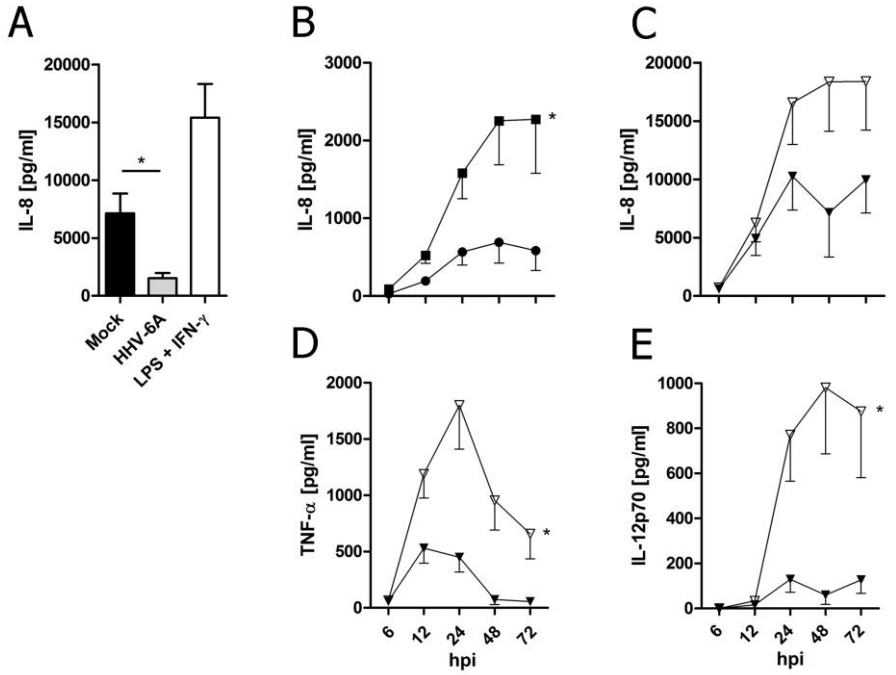

Figure 10. Replication competent HHV-6A (0.01 MOI) (grey or filled circles) reduced IL-8 secretion by immature DC at 3dpi compared to mock (black) (A) and also compared to UV inactivated HHV-6A (0.01 MOI) (filled squares) (B). In the presence of LPS and IFN-γ HHV-6A (0.01 MOI) (open triangles) slightly accentuated the secretion of IL-8 compared to medium only (filled triangles) (C), and also of TNF (D) and IL-12p70 (E). In panels A and B, DC where inoculated with HHV-6A in non-UV or in UV-inactivated form or with mock as negative control for three hours before they were washed and cultured in complete RPMI. In panels C, D and E, DC were cultured in complete RPMI supplemented with LPS and IFN-γ after the washing step. The cytokines were measured using CBA and data is shown as mean results (± SEM) for four donors and analyzed using two-way ANOVA for all panels except panel A were data is shown as mean results (± SEM) for at least seven donors and analyzed using Student's t-test. *$p<0.05$.

4.2.4 Suppressed allostimulatory capacity

Even though DC seem to be at least partly activated upon HHV-6A exposure, inoculation by the virus suppressed the capacity of DC to stimulate allogenic CD4+ T cell proliferation, as seen with MLRs (figure 11A and 11B). Interestingly this effect was further increased when the virus had been UV-treated prior to inoculation (figure 6B), indicating that the suppressed allostimulatory effect is independent of virus replication. These data are supporting two previous studies [144, 173] where DC were infected *in vitro*, and furthermore analogous with the data from an *in vivo* study where the leucocyte counts were significantly decreased in children with primary HHV-6 infection compared to in non-infected children [256]. Previous studies suggest that HHV-6A and HHV-6B can induce accumulation of p53 and that at least HHV-6B can protect the infected cell from apoptosis [257], indicating that an active replication might rescue the cells from apoptosis. However, inoculation with UV-inactivated virus in paper II of this thesis induced cell death to a similar extent as did inoculation with non-UV-inactivated virus at three dpi (figure 11C) suggesting that HHV-6A replication did not rescue the cells from apoptosis in this setting.

CBA targeting the Th1 associated cytokines IL-2 and IFN-γ and the Th2 associated cytokines IL-4, IL-6 and IL-10 suggested that a skewing towards a Th2 pathway was induced upon HHV-6A exposure (figure 11D). However, the effect was quite modest and a review of the literature suggests that this is usually the case upon HHV-6A infection. *In vitro* infection of PBMC induced both IFN-γ and IL-10 [258] and *in vivo* cytokine measurements in the serum of primary HHV-6 infected children revealed that the Th1/TH2 balance seems to tipped in neither direction [259].

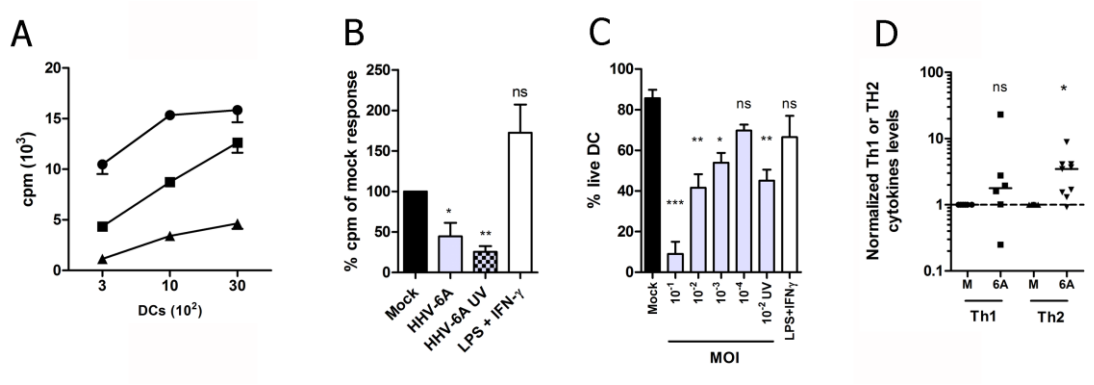

Figure 11. HHV-6A inoculation of DC suppresses their capacity to stimulate allogenic CD4+ T cell proliferation in mixed lymphocyte reactions (A and B). HHV-6A infection induces DC cell death (C) and skewing towards a Th2 pathway upon co-culture with allogenic T cells (D). In panel A, representative results for one of four donors are shown and data points are mean results (± SEM) of counts per minute (cpm) for triplicate wells. In panel B cpm mean results (± SEM) of at least four DC donors expressed as percentage in cpm responses compared to mock stimulated DC are shown. In panel C the bars represent the mean results (± SEM) of two to five donors. In panel D the results of CBA targeting IL-2, IL-4, IL-6, IL-10 and IFN-γ of supernatants from DC from three donors exposed to HHV-6A for three days prior to co-culture with allogenic T cells are shown. Data points represent the cytokine levels from HHV-6A (6A) and mock (M) exposed DC, normalized to mock. The IL-2 and IFN-γ constitute the Th1 group and IL-4, IL-6 and IL-10 the Th2 group. Median values for each group are marked with solid black lines. The data were analyzed using paired t-tests for panels B and D and unpaired t-test for panel C. *p<0.05, **p<0.01, ***p<0.001, ns: not signicifant.

4.2.5 Summary and conclusions

Taken together the results of paper II demonstrate that HHV-6A cannot replicate productively in DC. The cells got activated upon inoculation as seen with up-regulation of HLA-ABC via autocrine IFN-α signaling, and up-regulation of HLA-DR and CD86. HHV-6A suppressed the secretion of IL-8 in immature DC but this effect was overridden when other stimuli such as LPS and IFN-γ were present. The presence of LPS and IFN-γ also led to augmented secretion of TNF-α and IL-12p70 in HHV-6A inoculated DC. Inoculation of UV-inactivated virus induced somewhat less IFN-α secretion and a smaller augmenting effect on IL-8 secretion suggesting that HHV-6A might be able to replicate at low levels that were not detected by IFA or Q-PCR. A complementary approach could have been to assess replication by reverse transcriptase (RT)-PCR targeting a number of HHV-6A genes transcribed at different stages of infection. This might give information on whether a subset of genes are transcribed that could influence functional mechanisms of the cell.

So, do the data of paper II favor the incorporation hypothesis? DC get partly mature and partly activated but the vital action of DC in antiviral immunity, to induce T cell proliferation, is impaired (figure 12). One could argue that a potential bias in the MLR experiments is that HHV-6A induced an increased and titer dependent cell death of DC compared to mock at 3 dpi, which is the harvest time-point (figure 11C). In the MLR experiments the DC are further cultured for four additional days in the presence of allogenic T cells before the MLRs are harvested. Even though the identical number of live DC were added for the different stimulations in the initiation of the MLRs, the HHV-6A infected DC might die to a larger extent than mock inoculated DC over the course of the MLRs, resulting in an impaired potential for the T cells to receive proliferative stimuli. Hence, the assumed HHV-6A induced "anti-allostimulatory effect" might very well just be a result of dying DC in the MLR cultures. However, the single previous study on allostimulation by HHV-6A infected DC by Smith *et al.* also report a suppressive effect of HHV-6A and they harvested their DC after 17 hours [173]. Together our report and the Smith report suggest that HHV-6A do not constitutes an adjuvant effect, at least not *in vitro*.

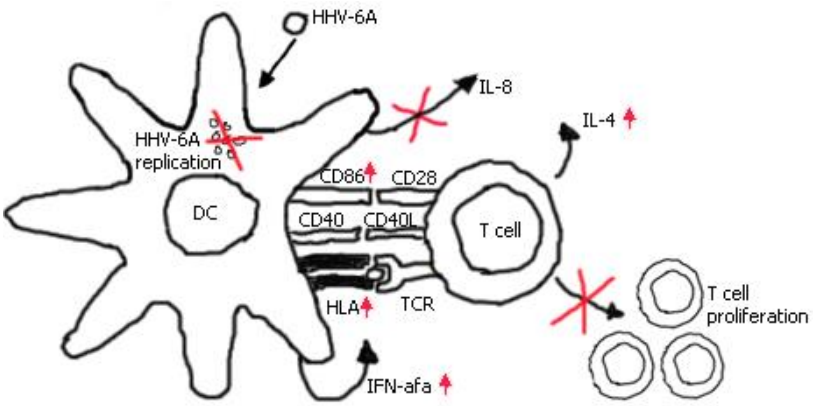

Figure 12. Schematic picture of the findings in paper II. Red crosses and up-pointing arrows represent inhibition and up-regulation respectively.

4.3 PAPER III

Many studies suggest an association between HHV-6 and MS. However, to date the total cumulative body of indications for a causal relationship provides only just that, an indication that HHV-6 might have a role in the cause or course of MS. One way to prove an HHV-6 etiology in MS would be if a clinical improvement could be observed after antiviral treatment targeting HHV-6. Patients who, during the course of disease have shown evidence of active viral infection or possibly a shift in anti-HHV-6 responses would be eligible for such a trial. To screen for these patients a robust, rapid and cost-effective method is required.

In paper III [260] we analyzed plasma and cerebrospinal fluid (CSF) using nested PCR. We found HHV-6 DNA in plasma and CSF in two and one of 27 possible MS patients respectively. Even though no viral DNA was found in any of the 33 headache patient controls the differences were not statistically significant. Hence, screening of cross-sectional samples for viral DNA at one time-point only does not seem to be the optimal approach to identify patients with active HHV-6 replication. Given the short periods of time when HHV-6 DNA is detectable longitudinal sampling would be preferred. In addition, serological screening for anti-HHV-6 IgM antibodies might be an easier measure of active replication.

Plasma and serum was used for analysis as it has the advantage of being easily accessible for sampling, but it might not reflect the viral status in the brain. CSF is a more accurate compartment when studying the brain [261] but is much more inconvenient to sample, for the clinician and of course even more for the patient. The aim of analyzing plasma and CSF samples in combination was to assess their concordance for HHV-6 DNA. None of the patient was positive in both plasma and CSF suggesting that local reactivations can occur. Nested PCR was chosen since it is cost-effective and rapid. However, it implies a risk for false positive results, so for a robust analysis it can be combined with sequencing of the PCR product to determine that a HHV-6 sequence has actually been amplified. Sequencing was not prioritized in paper III due to the low number of positive samples. Another drawback with nested PCR on plasma or serum samples is the risk of detecting viral DNA that has leaked out from lysed PBMCs. However, sensitivity is more important in screening approaches. Subsequently additional assays with higher specificity can be applied such as Q-PCR could have been applied if a higher frequency of HHV-6 DNA positive samples had been detected. However, given the negative results of paper III we did not find this procedure necessary. To conclude, given the low frequency of positive findings in this study screening for HHV-6 DNA in a cross-sectional setup does not seem to be an appropriate approach to identify MS patients where the virus might influence the disease.

4.4 PAPER IV

Genetic as well as environmental factors have been suggested to contribute to MS susceptibility. The strongest genetic risk allele for MS disease onset is *HLA-DRB1*15* [11, 12, 19], and *HLA-A*02* is a protective allele [21, 22]. Suggested environmental factors include low vitamin D levels [262], smoking [43] and various infections agents [263] such as HHV-6. Female sex is also a risk factor as more women than men are affected. Most studies have been focused on either genetics or environment, and many studies have investigated one risk factor only. However, interactions between these may occur. Therefore, focusing on HHV-6 biology, the aim of paper IV was to characterize the anti-HHV-6 IgG response in MS patients and healthy controls, and to assess if factors that can influence MS susceptibility can also affect the serological response against HHV-6.

The serum anti-HHV-6 IgG seroprevalence was 90%. No difference between MS patients and healthy controls was observed. This topic is controversial as different studies where whole virion lysate was used as antigen as some see an increased anti-HHV-6 IgG antibody titers in MS patients compared to controls [264-266], whereas others do not [200, 207, 267, 268]. Our study is the largest and together with the other negative studies it sets the controversy in favor for the notion that an increased IgG response against whole HHV-6 virions is not regularly seen in MS. However, it does not rule out the role for HHV-6 in MS. As the serological response against different HHV-6 proteins can differ [202] it is possible that a low response against a certain protein can be compensated by a higher response against another. Therefore investigation of individual proteins might be more informative. The central tissues in the pathophysiology in MS are within the CNS. However, sampling of serum or plasma is considerably more convenient than CSF and therefore investigations of relevant markers in the periphery of HHV-6 infection relevant in MS are warranting.

No difference in anti-p41 IgG levels was seen between MS patients and controls. As for antibody responses against HHV-6 lysate different studies report contradictory results [191, 202, 269]. The discrepancy might explained by the different assays used that applied antigens purified by different methods. Interestingly, serological in CSF responses against HHV-6A have been shown to be more abundant and antibodies of higher titers compared to HHV-6B suggesting a predominant role for HHV-6A in the CNS in MS disease [201]. A more detailed investigation assessing the HHV-6A or 6B species specific responses in the relatively large material that constitute this study would be very interesting. However, for a plausible performance it would require a convenient assay such as ELISA.

IgG levels might be regarded as a sign of history of infection. An increased frequency of reactivations of HHV-6 has been seen in RRMS during relapses [15, 16]. As incerased serum IgG titer against HHV-6 have been shown to positively associates with relapse risk in MS [212] an incerased frequency of reactivations might influence the MS disease and furthermore be reflected by an accumulating IgG levels in serum. However, we did not see this. Instead, the level of antiviral IgG antibodies against a

common virus such as HHV-6 might be interpreted as a reflection of a general activity that is influenced by various factors. Such factors include HLA types, serum vitamin D levels tobacco smoking and gender. As the anti-HHV-6 IgG response did not differ between MS patients and controls they could be grouped together in the analyses.

Interestingly, carriership of the MS protective allele *HLA-A*02* and tobacco smoking was associated with lower anti-HHV-6 IgG levels ($p<0.001$ and $p<0.02$ respectively), whereas females sex was associated with higher levels ($p<0.02$) (figure 13). No influence was seen for carriership of the MS risk allele *HLA-DRB1*15* or for serum vitamin D levels. It is well known that CD8+ cytotoxic T cell mediated immunity is very important in viral infections. With the interaction to HLA class I molecules on infected cells it is not surprising that we see an association to a *HLA class I* genes and not to a *HLA class II* gene. One mechanism underlying the protective effect of *HLA-A*02* against high levels of anti HHV-6 IgG antibodies might be its ability to present peptides without loading via the transporter associated with antigen processing (TAP) complex [270]. Both EBV and CMV encode for proteins that can block the TAP-mediated peptide loading on major histocompatibility complex (MHC) class I [271, 272]. If HHV-6 has a protein with a similar function then a lower people carrying HLA-A*02 might handle the primary infection and/or reactivations more efficiently. This could serve as a mechanistic explanation if increased anti-HHV-6 IgG levels can be utilized as a reflection of the history of the magnitude or frequency of an infection or reactivation. Hence, whereas HLA-A*02 might protect against HHV-6 mediated MS onset, providing a possible explanation for its protective effect against MS, HLA-DRB1*15 might present myelin peptides efficiently and thereby contribute to disease.

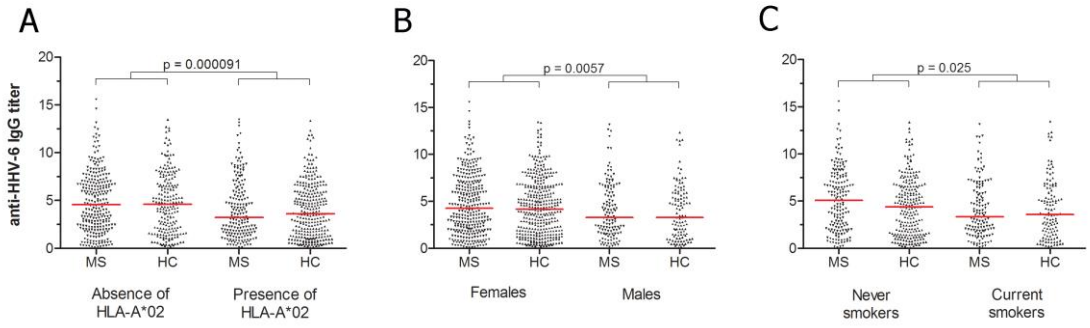

Figure 13. Anti-HHV-6 IgG antibody response in MS patients (MS) and in healthy controls (HC), divided by *HLA-A*02* carriership (A), gender (B) and smoking habits (C). Each dot represents one study participant. Median titers are marked with red lines. P-values are calculated using Mann Whitney U Test.

Female gender was associated with higher anti- HHV-6 IgG levels, possibly reflecting a more active serological immunity compared to males. Furthermore, this study revealed that smoking lowered the antibody response against HHV-6, which could be explained by a decrease in general serum IgG by tobacco smoking [273].

In conclusion, although no difference was seen in IgG response against HHV-6 between MS patients and controls, the serology measured was associated with factors previously associated with MS susceptibility. This indicates that an IgG response against a common virus like HHV-6 might be regulated by similar factors that also regulate susceptibility to autoimmune diseases like MS.

4.5 PAPER V

As HHV-6 is a ubiquitous virus that can establish lifelong latency in the host, factors like host genetics might play important roles in regulating anti-HHV-6 immune responses, which could have consequences for the establishment of latent infection and reactivation. The aim of this study was to investigate the influence of host genetics on the serological response against HHV-6 in the MS patients and healthy controls measured in project IV.

As no difference in anti-HHV-6 IgG status or levels was detected the patients and healthy controls were assembled in the analyses. All study subjects had been genotyped with nearly 500 000 SNP markers over the entire genome. The allele of each SNP was analyzed for association to antibody status or levels using logistic or linear regression analysis. Given the continuous and even distribution of anti-HHV-6 IgG levels seen in the study population (figure 13, paper IV) the cut-point level seemed somewhat arbitrary. Therefore, the results of the linear regression analysis might be more robust and reliable. Initially, the SNPs within the HLA region were used to impute the HLA alleles and investigate associations to antibody status or levels. No allele was associated to status but the association between *HLA-A*02* and lower antibody levels ($p=0.004$, $\beta=-0.123$) found in paper IV was found also using SNP imputation only instead of classical HLA typing as was primarily used in paper IV. A discussion on possible mechanisms for this association is given in section 4.4. *HLA-DQA1*05* was also associated to serological response against HHV-6 but to higher antibody levels ($p=0.018$, $\beta=0.105$). Whereas the biological characteristics of the HLA-A*02 molecule is well known less is known about the HLA-DQA1*05 molecule. Upon vaccination against Measles virus *HLA-DQA1*05* carriers respond with lower serum IgG antibody levels [274]. One possible and straight forward explanation for the association between higher levels of antibody against HHV-6 in *HLA-DQA1*05* carriers is that the HLA-DQA1*05 molecule have high affinity for certain HHV-6 peptides and thereby can facilitate efficient peptide presentation by B cells and thus higher antibody production. However, the binding of HHV-6 peptides to HLA-DQA1*05 molecules remains to be experimentally investigated.

In further linear (figure 14A) and logistic regression (figure 14B) analyses the HLA region was included as a covariate. Interestingly, the linear regression analysis revealed suggestive association ($p<1*10^{4}$) to a SNP (rs17162994) within 50 kilo base pairs (kb) from the *TRBV5-1* (T cell receptor beta variable 5-1) gene. Clearly, the TCR is a central molecule in both cellular and humoral immunity and given the abundant presence of αβ T cells over γδ T cells in circulation, the αβ T cells seem to be the lineage that is most linked to B cell help and in turn to antibody production. Therefore, an association between antibody responses against a common virus such as HHV-6, and a locus in the TCR β chain is not unexpected. The αβTCR is important also during T cell development. Assuming that the magnitude of primary infection and the magnitude and/or frequency of reactivations of HHV-6 are reflected by the antiviral IgG antibody response, an efficient cellular immune response by CTLs, which can control the infection, might be reflected in lower serum anti-HHV-6 IgG levels. The TCR has a

central role also in cellular immunity and therefore, the suggestive association seen between anti-HHV-6 IgG levels and an allele of the *TRBV5-1* gene might constitute a negative image of the antiviral cellular immunity.

Three additional genes with potential impact on Th cell development and Th1 or Th2 skewing were identified with suggestive association to anti-HHV-6 IgG levels: *CMIP* (rs2966097), *RUNX1* (rs2186290) and *MAML3* (rs6835277), and one with suggestive association to anti-HHV-6 IgG status: *KSR2* (rs10850917, rs7295136 and rs17619337). Together, the findings suggest that the magnitude of anti-HHV-6 IgG antibody secretion is affected by host genes with potent impact for T cell development and Th1 or Th2 skewing. As the association to the *HLA-DQA1*05* allele, neither these are unexpected finding. As the readout of the viral assay is the antiviral serological response it is likely that associations are seen to genes with impact on antiviral immunity.

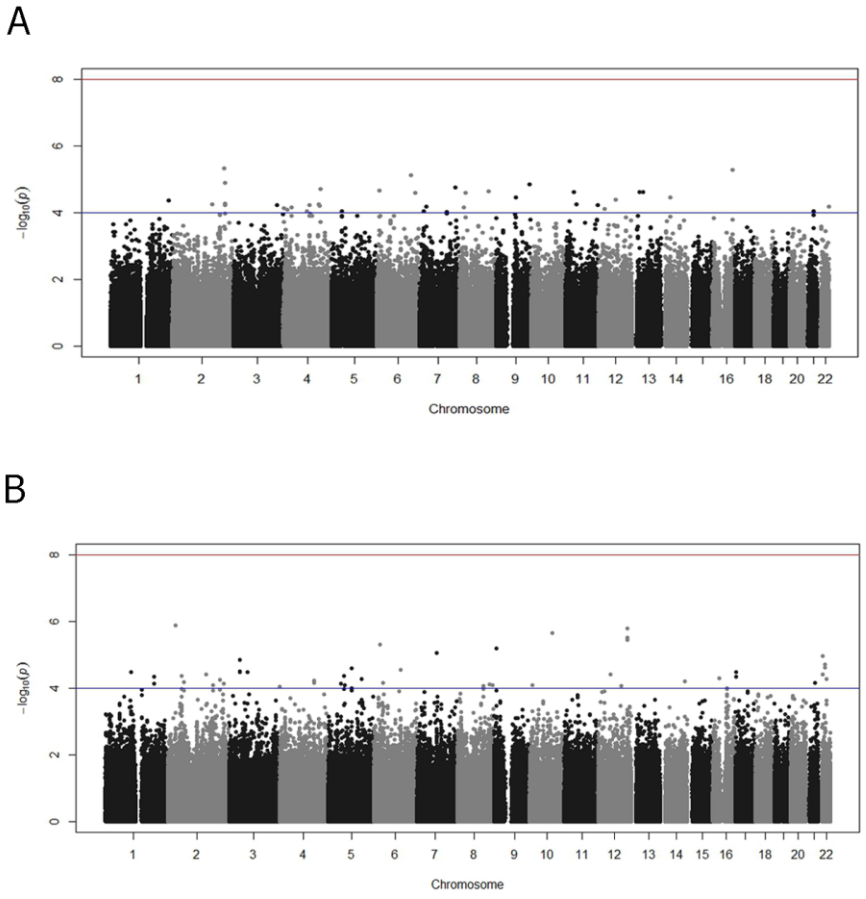

Figure 14. Manhattan plot with p-values from the linear (A) and logistic (B) regression analyses of GWAS markers, investigating association to anti-HHV-6 IgG nOD levels or status, respectively. *HLA-A*02* and *HLA-DQA1*05* were included as covariate in the linear regression analysis. Each dot represents the p-value of one marker, the lower horizontal line indicates the threshold for suggestive association $p=10^{-4}$ and the upper horizontal line $p=10^{-8}$, the threshold for GWAS association is $p=5*10^{-8}$.

To conclude, in this study we report for the first time results of a GWAS approach in HHV-6 infection. Furthermore we report a novel HLA allele with suggestive association to higher anti-HHV-6 IgG levels, *HLA-DQA1*05*. Furthermore, this study

provides several non-HLA genes with potential impact on serological immunity against HHV-6. Interestingly, a gene coding for a variable part of the TCR β chain was identified together with several genes with importance for skewing the Th cell population into Th1 or Th2 lineages. To investigate functional SNPs within the same haplotype blocks as the SNPs identified re-sequencing of the region of interest could be used. Even though none of the non-HLA markers reached the GWAS significance threshold further investigations in a larger material are warranting.

5 CONCLUDING REMARKS

To summarize the biological findings and methodological advances in this thesis:

1. Q-PCR read-out of $TCID_{50}$ is more robust than established methods. As different approaches yield different results on the same virus batch, the titration methods used within the HHV-6 field should be harmonized.
2. HHV-6A cannot replicate productively in DC but induce partial activation as seen by IFN-α mediated up-regulation of HLA-ABC, and up-regulation of HLA-DR and CD86. HHV-6A suppresses the IL-8 secretion and capacity to stimulate T cells, but augments IL-12p70 and TNF secretion.
3. Active replication of HHV-6 is not seen more often in MS patients than in controls, or at least, is difficult to detect in cross sectional samples.
4. The presence and levels of anti-HHV-6 IgG antibodies does not differ between MS patients and healthy controls, and the seroprevalence is 90%. However, anti-HHV-6 IgG antibody levels are associated with *HLA-A*02* carriership, smoking and gender, factors all linked to MS susceptibility.
5. Anti-HHV-6 IgG levels are significantly associated with *HLA-DQA1*05* carriership. Furthermore anti-HHV-6 IgG status and levels show suggestive association to a locus in the gene encoding the TCR beta variable chain and to genes with importance for steering the Th cell population into Th1 or Th2 lineages.

As exemplified from different angles throughout this thesis the support for an immune component in MS is overwhelmingly strong. The action of our immune system is specific and requires an activation signal. As discussed above the host cell protein incorporation hypothesis for tolerance breakage and MS induction, which was the starting point of this thesis, fulfills these two criteria. However, it implies other problems.

Both HHV-6A and HHV-6B have the capacity to infect oligodendrocytes, but the release of free virions is not commonly seen [150, 275, 276]. However, HHV-6A can induce vigorous apoptosis of a human oligodendrocyte cell line *in vitro* [154]. Evidences for a productive infection of oligodendrocytes with the release of cell free virions, is a vital step in the incorporation theory that needs to be clarified. Furthermore, these virions should be shown to contain myelin proteins. Both HHV-6A and HHV-6B [150, 277], can induce cell-to-cell fusion which seems to be a mechanism for viral spread [146, 183], therefore the requirement of cell free virions for this hypothesis might be somewhat problematic to meet. The notion of limited amounts of cell free virions in HHV-6 cultures is also supported by my personal lab experiences on virus propagation. Even though optimal cell lines were used, harvest of cell free supernatant yielded an infectious batch in around 10% of cases. In addition, given the numerous immune evasion strategies employed by HHV-6 it seems unlikely that the HHV-6 virion would constitute a potent adjuvant. In paper II we showed that HHV-6A exposed DC have hampered capacity to activate allogenic T cells. This effect was replication independent as UV treated virus had the same effect.

So what does this mean, that autoimmunity is not the major driving force in MS? An interesting discrepancy between EAE models and MS is that whereas myelin specific T cells are found at higher frequencies in EAE animals compared to healthy animals, this is not seen in MS, compared to controls, even though myelin-specific T cells from MS patients are of a more pro-inflammatory character [18]. It could be argued that feed-back mechanisms are at play in MS disease shutting down the immune reaction after an attack, which results in the unaltered frequencies. But it seems likely that if autoreactive immune attacks are the primary event in MS then the frequencies of myelin specific cells should be increased, given that myelin proteins are major targets.

An alternative explanation to a primary autoimmune attack, and to the host cell protein incorporation hypothesis, is that the mechanism of action in the onset of MS pathogenesis is provided by the HHV-6 infection itself. However, it does not seem to be as clear-cut as the host cell protein incorporation hypothesis assumes. Instead of buddying of myelin protein containing virions, the onset of events is more likely to start with a lytic infection of oligodendrocytes leading to the upregulation of MMPs, which enhance influx of leucocytes. Even though the influx of leucocytes into the CNS is dependent also on the interaction between the activation induced molecule VLA-4 on leucocytes and VCAM-1 on the capillary endothelial cells, it seems likely that virus specific leucocytes can infiltrate CNS tissues to screen the tissue for local reactivations as neurotropic viruses such as HHV-6 can reactivate locally in the CNS [260, 278]. Therefore, the criteria of peripheral activation might not be that strict.

To conclude, the discussion on virus induced MS onset reveals that a comprehensive theory of a mechanism for MS onset is very difficult to pose with pitfalls all over. Instead of designing *in vitro* experiments to prove a specific hypothesis, my position is that research on a virus etiology of MS should be performed closer to the patients. As discussed in section 1.3.4 a randomized, placebo-controlled and double blinded clinical trial with an efficient anti-HHV-6 drug should be conducted on MS patients in the search for a proof of principle. Firstly, the prodrug for ganciclovir VGCV has proven to be safe in the treatment on CFS patients [227] and ganciclovir have well documented efficacy against HHV-6 [224]. However, the specific efficacy of VGCV against HHV-6 should be investigated further before starting a trial. Secondly, CIS patients may be a good study group as HHV-6 has a suggested role primarily in early events of MS. Thirdly, if HHV-6 is important for the pathogenesis in only a subset of MS patients, those with a serological response in the CNS, suggesting a local CNS reactivation, are probably the ones that would benefit from antiviral treatment. And finally, a reliable virological method should be developed and used to assess the effect on the virus by the treatment. A marker of CNS infection that can be detected in peripheral blood would be desirable as blood is much more convenient to sample than CSF. If VGVC treatment of CIS patients reduces the HHV-6 load and induces a clinical improvement, then the time has come to investigate mechanisms that may underlie a HHV-6 induced pathogenesis of MS.

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